Adenosine Receptor Signaling

and the Activation of Mitogen-Activated Protein Kinases

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abstract

The nucleoside adenosine is present in all cells and body fluids of all living organisms; its production, both intracellularly and extracellularly, is tightly coupled to energy consumption resulting in increasing extracellular adenosine levels with increased energy consumption. Adenosine is metabolized by adenosine deaminase and adenosine kinase. Deamination leads to the production of inosine, which appears in concentrations similar to those of adenosine. Adenosine exerts modulatory effects on many body functions and is known to regulate, for example, the cardiovascular and central nervous system, pain modulation, mast cells and immune function. These physiological effects are mediated by four pharmacologically and biochemically distinct adenosine receptors, which belong to the family of G protein coupled receptors activating heterotrimeric G proteins. The adenosine A_1 and A_3 receptors generally couple to G_i proteins, whereas the adenosine A_{2A} and A_{2B} receptors activate G_s proteins.

In order to describe adenosine receptor pharmacology and signaling the recombinant human adenosine receptors were stably transfected into Chinese hamster ovary (CHO) cells, which normally do not express adenosine receptors. This is of advantage for the description of single adenosine receptor subtypes since sufficiently specific pharmacological tools are not available and since most cells express more than one adenosine receptor subtype. Furthermore, this recombinant cellular system allows the comparison of the human adenosine receptors in front of a similar cellular background.

Human adenosine receptors are, indeed, activated by adenosine, whereas its metabolite inosine is only active at the adenosine A_1 and A_3 receptor, and even there inosine has low potency and efficacy. Thus, adenosine is the main ligand for adenosine receptors. Moreover, adenosine A_1 and A_{2A} receptors may be constitutively activated at basal adenosine levels (30 – 300 nM), whereas the A_{2B} and A_3 receptor are only activated when adenosine levels are increased. Inosine – at high concentrations – may have effects at the adenosine A_3 receptor, at least where it is highly expressed.

Adenosine affects cell growth positively and negatively. An important role in mitogenesis is played by the family of mitogen-activated protein kinases (MAPK) and we show that all adenosine receptors expressed in CHO cells mediate signaling to the MAPK extracellular signal-regulated kinase 1/2 (ERK1/2). ERK1/2 phosphorylation is time- and dose-dependently increased upon stimulation with the unspecific adenosine receptor agonist 5´-N-ethylcarboxamidoadenosine (NECA) and with the endogenous ligand adenosine. Adenosine receptors showed different efficacy in mediating ERK1/2 phosphorylation: $A_3 > A_1 > A_{2A} > A_{2B}$. The EC_{50} value for the adenosine A_1, A_{2A}, and A_3 receptor mirrored that for effects on e.g. adenylyl cyclase, but NECA-induced ERK1/2 phosphorylation in CHO A_{2B} cells was half-maximal already at 20 nM, compared to a half-maximal cAMP production at 1.4 µM.

Due to the high efficacy and the high potency of agonists to induce ERK1/2 activation via the adenosine A_3 and A_{2B} receptor, respectively, we specifically examined these intracellular signaling pathways, using both pharmacological and molecular biological tools. Pharmacological tools such as kinase and adenylyl cyclase inhibitors, however, caused unexpected problems. Due to structural similarity, inhibitors binding to the ATP-binding site of such enzymes may also bind to and block the ligand-binding site of adenosine receptors, which could be shown by competitive radioligand binding experiments.

The adenosine A_3 receptor signals to ERK1/2 by the release of $\beta\gamma$ subunits from pertussis toxin-sensitive G proteins, activation of phosphatidylinositol-3′-kinase (PI3K), the small GTP-binding protein Ras and the MAPKK MEK. The G_s-coupled adenosine A_{2B} receptor, on the other hand, accomplishes ERK1/2 activation in a cAMP-dependent but cAMP-dependent protein kinase (PKA)-independent manner. Furthermore, this pathway uses PI3K and transactivation of the epidermal growth factor receptor, whereas activation of the small GTP-binding protein Rap1 is not necessary for ERK1/2 activation. Signaling to the cAMP response element binding protein (CREB) and the stress-activated protein kinase p38 involves cAMP and PKA – independent of PI3K.

The molecular aspects of adenosine receptor signaling *in vivo* are still obscure, but the knowledge of intracellular signaling pathways activated by single adenosine receptor subtypes in recombinant cell systems may present some support for mechanisms in the *in vivo* situation.

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to my father

papers

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abbreviations

AKAP - A Kinase (PKA) anchoring proteins
BAPTA-AM - 1,2-bis(2-aminophenoxy)ethane-N,N,N´,N´-tetraacetic acid tetrakis (acetoxymethyl ester)
βARK-ct - β-adrenergic receptor kinase – C-terminus
cAMP - cyclic adenosine-3′,5′-monophosphate
CGS15943 - 9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine
CGS21680 - 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine
CHO cells - Chinese hamster ovary cells
CPA - N^6-(cyclopentyl)-adenosine
CRE - cAMP response element
CREB - cAMP response element binding protein
CTX - cholera toxin
$[Ca^{2+}]_i$ - intracellular Ca^{2+} concentration
DAG - diacylglycerol
DPCPX - 1,3-dipropyl-8-cyclopentylxanthine
ERK1/2 - extracellular signal-regulated protein kinase 1/2
Epac - exchange factor protein activated by cAMP
GEF - guanine nucleotide exchange factor
GPCR - G protein-coupled receptor
Grb2 - growth factor receptor-bound protein 2
GST - glutathione-S-transferase
H89 - N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide
IB-MECA - N^6-(3-iodobenzyl)-N-methyl-5'-carbamoyladenosine)
JNK - c-jun-N-terminal kinase
LY294002 - 2-(4-morphonilyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride
MAPK - mitogen-activated protein kinase
MAPKK - MAPK kinase
MAPKKK - MAPKK kinase
MBP - myelin basic protein
MEK - MAPK/ERK kinase
NECA - 5´-N-ethylcarboxamidoadenosine
NBMPR - nitrobenzylthioinosine
p38 - stress-activated protein kinase, 38 kDa
PKA - cAMP-dependent protein kinase
PKB/ Akt - protein kinase B
PKC - Ca^{2+}-dependent protein kinase
PLC - phospholipase C
PTX - pertussis toxin
RTK - receptor tyrosine kinase
SAPK - stress-activated protein kinase
Shc - Src homology/collagen protein
Sos - the product of the mammalian homolog of son of sevenless gene
SQ22536 - 9-(tetrahydro-2´-furyl) adenine
PAGE - polyacrylamide gelelectrophoresis
PD98059 - 2´-amino-3´-methoxyflavone
PDBu - phorbol-12,13-dibutyrate
PI3K - phosphatidylinositol-3′-kinase
PP2 - 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4]pyrimidine
PP3 - 4-amino-7-phenylpyrazol[3,4-d]pyrimidine
Raf - mammalian gene product of the raf oncogene
Rap1 - Ras-related protein 1, Ras proximate
Ras - mammalian gene product of the ras oncogene
RasS17N - dominant-intefering mutant of Ras with decreased affinity for GTP
Ro-318220 - 3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide methanesulfonate
SCH58261 - 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine

introduction

Short introduction to the physiology and the metabolism of adenosine

The endogenous nucleoside adenosine plays a central role as a structural element of nucleic acids and in the energy metabolism of all living organisms. Adenosine's role as a sensor of the metabolic state and as a general modulator of biological functions in a variety of cell types, tissues, organs and physiological processes has been acknowledged for a long time, even though the underlying cellular and intracellular mechanisms remain obscure. The physiological effects of adenosine were first described in the cardiovascular system and gastrointestinal tract (Drury and Szent-György, 1929) but a much broader spectrum of modulatory functions has been described today. Adenosine plays a major role in the cardiovascular system (Baines et al., 1999), in the central nervous system (Fredholm, 1995b, 1997; Sebastiao and Ribeiro, 1996; Svenningsson et al., 1999; Haas and Selbach, 2000), as an endogenous pain modulator (Sawynok, 1998, 1999), in the gastrointestinal tract (Roman and Fitz, 1999), the immune system (Cronstein, 1994), mast cell degranulation (Linden, 1994; Marquardt, 1998), asthma (Forsythe and Ennis, 1999), cell growth and apoptosis (Abbracchio et al., 1997a; Ohana et al., 2001), etc.

The main focus of this thesis, however, is the actions of adenosine on a cellular and molecular level. For more information on the macroscopic physiological role of adenosine the reader is referred to the above-mentioned reviews.

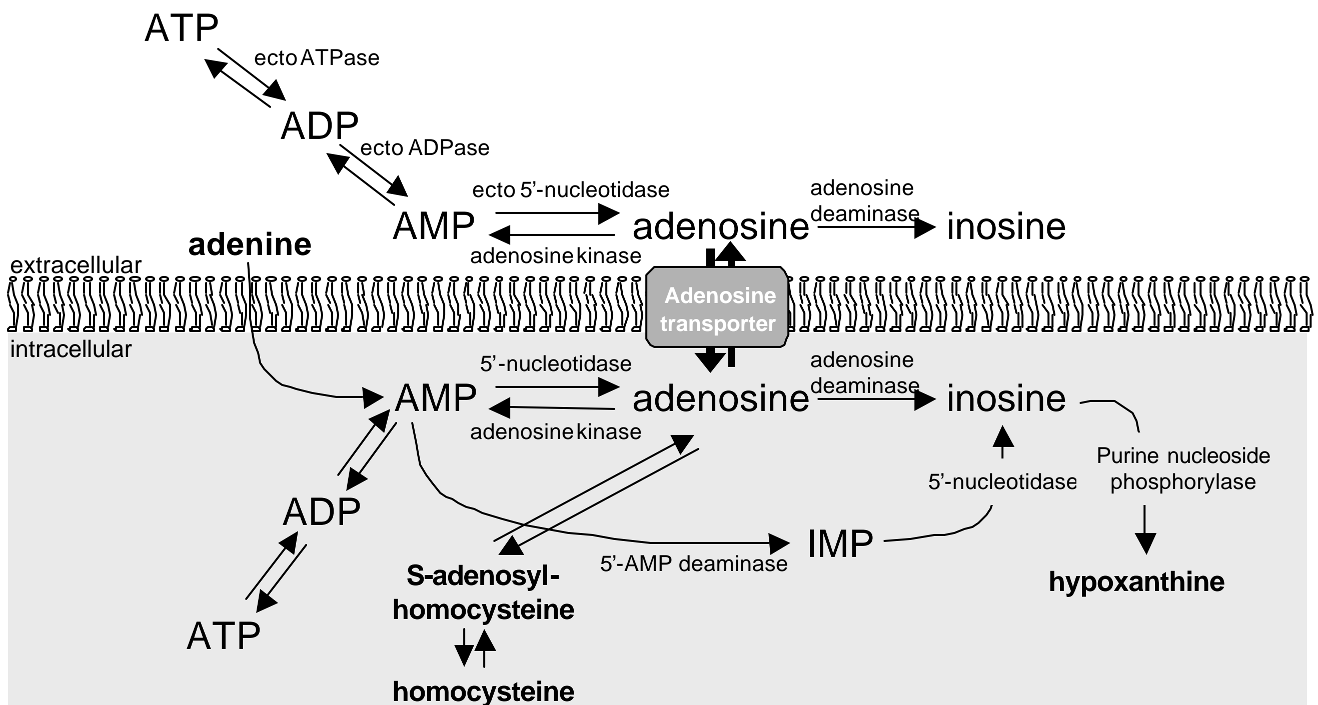

Fig. 1: Schematic summary of the regulation of extra- and intracellular adenosine and inosine concentrations. (AT(D, M)P, adenosine tri-(di-, mono-) phosphate; IMP, inosine monophosphate).

Intracellular adenosine is produced (Fig. 1) either by breakdown of adenosine 5′ phosphates (AMP, ADP, ATP) – a reaction involving 5′ nucleotidase – or by hydrolysis of S-adenosylhomocysteine. Extracellular adenosine is either produced by fast nucleotide hydrolysis catalyzed by ecto-ATPase, ecto-ATP-diphosphohydrolase and ecto-5′ nucleotidases (Zimmermann et al., 1998) or it originates from the intracellular pool, which stands in close contact with the extracellular space by means of bi-directional equilibrative and Na^+-dependent concentrative nucleoside transporters (Williams and Jarvis, 1991; Anderson et al., 1996). Metabolism of adenosine by phosphorylation or degradation to inosine is catalyzed by adenosine kinase and adenosine deaminase, respectively (Arch and Newsholme, 1978; Lloyd and Fredholm, 1995). Adenosine deaminase does not only metabolize adenosine but also, and with greater efficiency, 2′-deoxy adenosine, which is a lymphotoxic product of the purine metabolism (Cristalli et al., 2001). Indeed, a genetic defect resulting in the lack of adenosine deaminase is associated with a severe combined immunodeficiency disease (SCID) in humans.

The concentration of free adenosine inside as well as outside the cell is closely related to energy consumption. One consequence is that adenosine concentration, which is kept rather constant (30 – 300 nM) under basal conditions (Ballarin et al., 1991), increases during metabolic stress such as muscle activity and nerve activity or under pathophysiological conditions such as hypoxia and ischemia, when extracellular levels of adenosine can reach concentrations of 10 µM or even higher (Hagberg et al., 1987; Dux et al., 1990).

The adenosine metabolite inosine follows the fluctuations of adenosine concentration closely, resulting in similar levels of adenosine and inosine most of the time (Zetterström et al., 1982; Hagberg et al., 1987; Ballarin et al., 1991). The two nucleosides are closely linked to each other by means of adenosine deaminase. Inhibitors of adenosine deaminase are used clinically in order to increase adenosine levels, which simultaneously lead to a decrease in inosine. Because inosine and adenosine concentrations are so closely related, both were suggested to play physiological roles. However, inosine can also be independently formed by direct hydrolysis of inosine monophosphate.

Adenosine receptors belong to the family of GPCRs

The effects of extracellular adenosine on cells are mediated by four different membrane-spanning adenosine receptors, which belong to the family of G protein-coupled receptors (GPCR).

Common features of G protein coupled receptors

The family of GPCRs is a large and functionally very diverse protein super-family, indeed one of the biggest protein families known, with over 1000 GPCRs described to date, and about 50 % of all therapeutics target these receptors (Flower, 1999). Furthermore, genes coding for GPCRs have been found in a number of DNA viruses (Rosenkilde et al., 2001). The diversity of this receptor family enables cells to respond appropriately to environmental stimuli as diverse as odorants, hormones, peptides, ions, small molecules, such as catecholamines and amino acids, and even photons.

GPCRs consist of a single polypeptide chain which traverses the cell membrane seven times with the N-terminus at the extracellular and the C-terminus at the intracellular face of the membrane (Fig. 2) and is thus similar in structure to

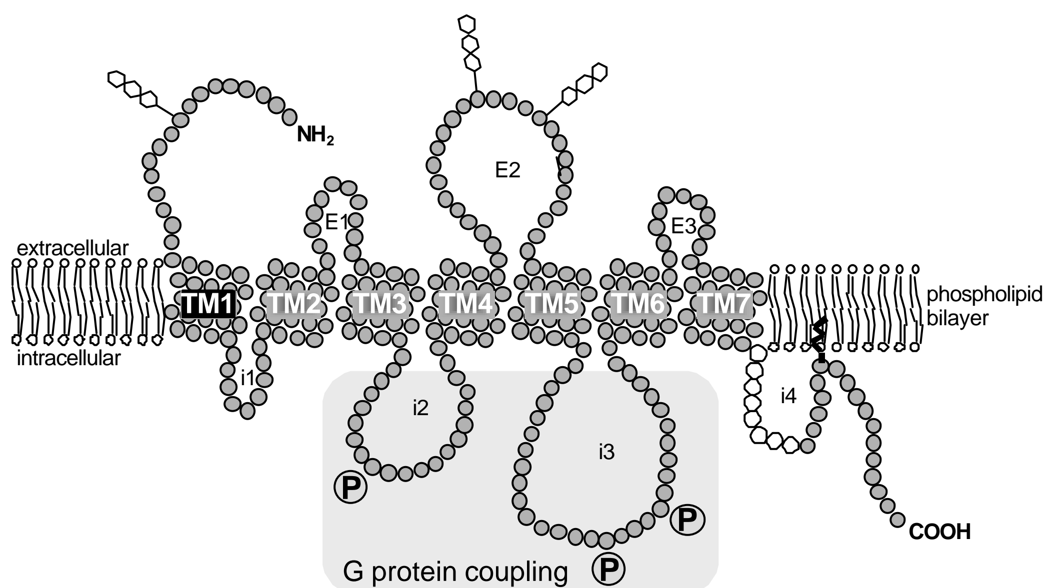

Fig. 2: Schematic drawing of the molecular features of G protein coupled receptors. TM1-7, transmembrane domain 1-7; i1-4 and E1-3 intracellular 1-4 and extracellular loop 1-3, respectively; P – putative phosphorylation sites; ⬡ - N-linked glycosylation; for more details see text.

rhodopsin (Dixon et al., 1986; Henderson and Schertler, 1990). Structural analysis of GPCRs has been hampered by poor crystallization of the purified receptor protein. Thus only indirect evidence is available indicating that the seven trans-membrane domains, which each contain 20 – 25 amino acids, form barrel-like α-helical structures perpendicular to the membrane, whereas the N- and C-termini and the three extra- and intracellular loops appear to be more flexible.
Crystal structures at high resolution of mammalian rhodopsin (Palczewski et al., 2000) and bacteriorhodopsin (Luecke et al., 2000) from *Halobacterium halobium* were described recently and basically confirmed the indirect structural data available previously. Both low-resolution structural information and the recently described high-resolution structures of rhodopsin/ bacteriorhodopsin and other GPCRs have been valuable for modeling adenosine receptor structures (IJzerman et al., 1992, 1994; van Galen et al., 1994; for a review see Fredholm et al., 2001b). GPCRs are glycoproteins, which are modified by N-linked glycosylation at specific Asn-Xaa-Ser/Thr motifs that are usually close to the N-terminal region. Specific cysteine residues in the C-terminal region are used to anchor the C-terminal tail to the membrane by palmitoylation.

The GPCR superfamily can be further divided into different classes or families characterized by their ligand binding domains (Bockaert and Pin, 1999; Gether, 2000). Family 1a receptors bind small molecules deep in the membrane in between the transmembrane domains. Family 1b receptors bind to small peptide ligands with binding domains at the N-terminus and on the surface of the receptor protein, and family 1c receptors bind large glycoproteins mainly with their N-terminal region. Furthermore, family 2 GPCR also bind peptide ligands with their N-terminal part, whereas family 3 receptors are able to sense very small ligands such as γ-aminobutyric acid (GABA), pheromones or Ca^{2+} within their huge N-terminal domain.
It is generally acknowledged that the conformational changes in the receptor structure induced by ligand binding involve the transmembrane helices 3 and 6, which protrude like a corkscrew into the

cytoplasm. These conformational changes transfer the signal across the membrane into the cytoplasm (Gether and Kobilka, 1998). Thereby, GPCRs activate heterotrimeric guanine nucleotide binding proteins (G proteins) (Gilman, 1987), which interact with specific domains on the intracellular face of the receptor (Hamm, 2001). The receptor's intracellular loops i2 and i3 are mainly responsible for receptor–G protein interaction, but also regions on the C-terminal tail of the receptor have been implied to mediate binding to G proteins (Franke et al., 1990; Ernst et al., 2000; Marin et al., 2000). In addition it was recently proposed that palmitoylation of the receptor partly determines the specificity of G protein coupling (Mokhopadhyay, 2002).

Heterotrimeric G proteins consist of three different subunits, the guanine nucleotide-binding α, and the β and γ subunits. β and γ subunits are tightly tethered by non-covalent interaction and thus build a functional unit, the $\beta\gamma$ subunit. About 23 different α subunits encoded by 17 different genes have been described, while five β and eleven γ subunits are known. The α subunits are grouped into four classes, the G_s, G_i, G_q and G_{12} (Gudermann et al., 1997).

G proteins are activated by GPCRs in their active conformation. When a GPCR is activated – e.g. by agonist binding – GDP bound to the inactive α subunit of the G protein is released, and the guanine nucleotides in the cytoplasm compete for the empty binding site. Although this site binds GDP and GTP with similar affinity, GTP is present at in at least 10 times higher concentrations and is therefore most likely to occupy the binding site. GTP binding induces dissociation of the G protein-receptor complex and also of the heterotrimeric G protein. The $\beta\gamma$ subunit and the α-GTP subunit, which both are anchored to the membrane by a lipid anchor, diffuse along the membrane in order to activate or inhibit an appropriate effector. The resulting signal is inactivated by hydrolysis of GTP to GDP by an intrinsic GTPase activity of the α subunit. The α-GDP subunit then again shows a high affinity for the (inactive) receptor and will - together with the $\beta\gamma$ subunit – re-establish the original heterotrimeric state, thus completing the cycle.

The different families of G proteins are able to stimulate or inhibit different effector systems. The first G proteins that were described in the classical experiments by Gilman (Northup et al., 1980) mediated the stimulation of cAMP production and, thus, were called stimulatory G proteins or G_s. The G_s family consists of two splice variants of the G_s gene, resulting in a $G_{s\ short}$ and a $G_{s\ long}$ protein, and the G_{olf} (olfactory) protein. The latter is primarily expressed in the olfactory epithelium and olfactory neurons, but is also found in the striatum (Jones and Reed, 1989). All of the G_s proteins can be activated by cholera toxin (CTX), which ribosylates the α subunit and thereby blocks the endogenous GTPase activity, preventing inactivation of the G protein.

Later, other G proteins were discovered that decrease cAMP levels by inhibition of adenylyl cyclase (AC): the inhibitory G_i class of G proteins. Based not only on functional but also on structural similarities this class consists of the subtypes G_{i1-3}, G_o (other), the two forms of the retinal transducin G_{t-c} (cones) and G_{t-r} (rods), the G_{gust} (gustatory) and G_z. All of these G_i proteins, except for G_z, are sensitive to the bacterial pertussis toxin (PTX) from *Bordetella pertussis*, which causes ADP-ribosylation of a specific cysteine residue thus preventing the dissociation of the α and $\beta\gamma$ subunit and, consequently, prevents activation of the G protein.

The G_q family consists of the members $G_{q, 11, 14, 15, 16}$. $G_{q/11}$ are ubiquitously expressed and their activation leads to an increase in

phospholipase Cβ (PLCβ) activity. The functions of $G_{14, 15, 16}$, however, are still obscure, but they also mediate PLC activation. Finally, the ubiquitously expressed G_{12} family, consisting of G_{12} and G_{13}, is believed to couple to Na^+, H^+ exchanger and to small GTP binding proteins (Offermanns and Simon, 1996). $G_{12/13}$ also play an important role in mediating cytoskeletal reorganization via the small GTPases Rac/Cdc42 and Rho (Dhanasekaran and Dermott, 1996).
Besides the task of mediating G_α-receptor interaction, βγ subunits are also able to affect intracellular signaling directly (Sternweis, 1994; Ueda et al., 1994; Clapham and Neer, 1997; Ford et al., 1998). βγ subunits regulate e.g. phospholipases (Rhee and Bae, 1997), ion channels (Ikeda, 1996; Schneider et al., 1997), AC I, II, IV (Sunahara et al., 1996), GPCR kinases (GRK) (Pitcher et al., 1992), and phosphatidylinositol-3′-kinase γ (PI3Kγ) (Vanhaesebroeck et al., 1997).

Even though most of the GPCRs generally couple to a single family of G proteins, it has become clear that there is a certain degree of promiscuity. The most famous example of this is the thrombin receptor (protease activated receptor, PAR), which has been shown to couple to $G_{q/11}$, G_i, $G_{12/13}$ proteins in the same cell (Gilchrist et al., 2001). The molecular basis for this, however, is not yet completely understood. It may be an intrinsic capability of certain GPCRs, or depend on posttranslational modification of the receptor (e.g. phosphorylation), on accessory proteins, or on the cellular background. Furthermore, the expression level of the receptor and/or the G proteins may be of importance since studies using overexpression of either protein have shown interactions, not all of which have been verified *in vivo*.
The β_2-adrenergic receptor is in fact able to switch G protein coupling from G_s to G_i upon phosphorylation via PKA (Daaka et al., 1997). There is also evidence that prostaglandin E receptors may switch from G_s to G_i coupling depending on PKC-mediated phosphorylation (Nordstedt et al., 1989). However, it is not clear whether this is a common mechanism for GPCR coupling.
Activation of GPCRs may lead to various degrees of desensitization and down-regulation of receptor expression, leading to tachyphylaxis, i.e. the receptor response to a substance is reduced. One can distinguish between desensitization of the receptor that is directly stimulated by the drug (homologous desensitization) and desensitization of other receptors than the drug acts on (heterologous desensitization). On the other hand, antagonist treatment may lead to sensitization and even to an increase in receptor number. When dealing with receptor desensitization it is important to differentiate between short-term effects and long-term effects. Short-term changes often depend on posttranslational modification of the receptor via phosphorylation (Lefkowitz, 1998). Receptor phosphorylation locks the receptor in an inactive state and induces formation of a complex including the receptor itself, β-arrestin and clathrin in clathrin-coated pits, thus leading to receptor endocytosis. This process of receptor uptake does not immediately reduce receptor number but it decreases the amount of receptor available for ligand binding. The engulfed vesicles reach endosomal compartments from which the receptor can either be recycled and re-embedded in the cell membrane or be degraded in late endosomes. Receptor degradation leads to a long-term desensitization due to an absolute decrease in receptor number (Ferguson and Caron, 1998).

Adenosine receptors

Adenosine receptors were postulated because of the observation that adenosine´s actions in the heart could be antagonized by caffeine (De Gubareff and Sleator Jr, 1965). The original classification of adenosine receptor subtypes in A_1 (R_i) and A_2 (R_s) receptors (van Calker et al., 1978, 1979) was based on the opposite effects of agonists on the levels of cAMP in diverse tissues: inhibitory and stimulatory effects for the A_1 and the A_2 receptors, respectively (Londos et al., 1980; Fredholm, 1982). Adenosine A_2 receptors were subdivided into adenosine A_{2A} and A_{2B} receptor (Daly et al., 1983) based on their ability to stimulate cAMP production in brain slices at low (0.1-1 µM) and high (≥ 10 µM) adenosine concentration, respectively. This classification was confirmed by ligand-binding assays and differential affinity towards adenosine derivatives, such as 2-(4-methoxyphenyl)adenosine, and molecular biological evidence (Bruns et al., 1986; Fredholm et al., 2001b). The most recently discovered adenosine receptor, the adenosine A_3 receptor, was identified through molecular cloning from a rat testis cDNA library and its sequence similarity to adenosine receptors (Zhou et al., 1992). During the past decade the four different adenosine receptors, the adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor, have been cloned from several species including man, mouse, rat, dog, sheep, rabbit, chick and guinea pig (Maenhaut et al., 1990; Libert et al., 1991, 1992; Meyerhof et al., 1991; Chern et al., 1992; Fink et al., 1992; Furlong et al., 1992; Olah et al., 1992; Pierce et al., 1992; Stehle et al., 1992; Townsend-Nicholson and Shine, 1992; Zhou et al., 1992; Salvatore et al., 1993; Sajjadi and Firestein, 1993; Ren and Stiles, 1994; Linden, 1994; Jacobson et al., 1995; Peterfreund et al., 1996) and described biochemically and pharmacologically (Fredholm et al., 2000, 2001b). The adenosine receptor subtypes A_1, A_{2A}, A_{2B}, and A_3 all belong to the family of GPCRs group 1a and, thus, show the heptahelical structure typical for these receptors. The genes for the four adenosine receptors are located on the human chromosomes 1, 22, 17 and 1, for the A_1, A_{2A}, A_{2B}, and A_3 receptor gene, respectively (MacCollin et al., 1994; Monitto et al., 1995; Townsend-Nicholson et al., 1995a-b; Le et al., 1996; Deckert et al., 1997). The proteins encoded by the A_1, A_{2A} and A_3 genes have a molecular weight of about 36.7, 36.4 and 36.6 kDa, respectively, whereas the adenosine A_{2A} receptor, due to its long C-terminal tail, has a molecular weight of 45 kDa (Palmer and Stiles, 1995). For comparison, the carboxyterminal tail of the A_{2A} receptor is 122 amino acids in man, guinea pig and dogs and 125 amino acids in rats but only 36 amino acids for the adenosine A_1 receptor.

The pharmacological tools available for the study of adenosine receptor were recently reviewed (Fredholm et al., 2001b). Generally, it can be concluded that ligands with high affinity and specificity are only available for the adenosine A_1 and A_{2A} receptors, whereas selective stimulation or blockade of adenosine A_{2B} and A_3 receptors in the presence of other adenosine receptors is generally not possible.

Adenosine A_1, A_{2A} and A_{2B} receptors bind to and are antagonized by methylxanthines, such as caffeine, theophylline or enprofyllin, whereas the adenosine A_3 receptor subtype is relatively xanthine-insensitive (Fredholm, 1995a; Fredholm et al., 1999; van Muijlwijk-Koezen et al., 2001), which may have been a reason for its relatively late discovery. All of the adenosine receptors naturally bind to adenosine, and the adenosine A_1 and A_3 receptors, may – under certain conditions

– be activated by the metabolite inosine (Jin et al., 1997, see also paper I). Information from the literature on the action of inosine at adenosine receptors is contradictory (Jones et al., 1981; Collis et al., 1986; Ethier et al., 1993), perhaps in part due to inosine's effects on the A_3 receptor (Jin et al., 1997; Tilley et al., 2000). Most of the references from the 1980s, when the existence of a xanthine-insensitive adenosine A_3 receptor was unknown, excluded adenosine receptors from the effects mediated by inosine (Jones et al., 1981), whereas later studies explain inosine effects mainly by the involvement of adenosine A_3 receptors (Jin et al., 1997; Tilley et al., 2000).

Just like other GPCRs adenosine receptors react to agonist exposure and activation by becoming internalized. Adenosine A_1 receptor uptake was shown in neutrophils (Falleni et al., 1999), an epithelial cell line (Gines et al., 2001), a pituitary cell line (Navarro et al., 1999), in DDT_1MF-2 smooth muscle cells (Ciruela et al., 1997; Saura et al., 1998), and in CHO cells transfected with the human adenosine A_1 receptor (Ferguson et al., 2000). The other adenosine receptors - A_{2A} (Mundell and Kelly, 1998; Mundell et al., 2000), A_{2B} (Matharu et al., 2001; Penn et al., 2001) and A_3 (Ferguson et al., 2000; Santini et al., 2000; Trincavelli et al., 2000; Macchia et al., 2001) – also internalize upon agonist exposure. This redistribution of adenosine receptor results in the desensitization of the cell system.

Adenosine receptors – tissue distribution

The tissue distribution of adenosine receptors has been investigated extensively (see Table 1). However, the knowledge about the adenosine A_{2B} and A_3 receptors is limited, partly because of

Table 1: Tissue distribution of adenosine receptors. Results are based both on detection of the receptor protein by radioligand binding, as well as detection of its mRNA by RT-PCR, northern blotting or *in situ* hybridization. Table slightly modified from Fredholm et al. (2001).

A_1 receptor	A_{2A} receptor	A_{2B} receptor	A_3 receptor
High expression Brain (cortex, hippocampus, cerebellum). lamina IIi/ spinal cord. Eye, adrenal gland, atria	*High expression* Spleen, thymus, leukocytes (both lymphocytes and granulocytes), blood platelets. Striatopallidal GABAergic neurons, olfactory bulb	*High expression* Cecum, colon, bladder	*High expression* Testis (rat), mast cells (rat)
Intermediate expression Other brain regions. Skeletal muscle, liver, kidney, adipose tissue, salivary glands, esophagus, colon, antrum, testis	*Intermediate expression* Heart, lung, blood vessels, peripheral nerves	*Intermediate expression* Lung, blood vessels, eye, median eminence, mast cells	*Intermediate expression* Cerebellum (human?), hippocampus (human?), microglia cells (mouse; Schulte et al., unpublished results), lung, spleen (sheep), pineal
Low expression Lung (but probably higher in bronchi), pancreas	*Low expression* Other brain regions	*Low expression* Adipose tissue, adrenal gland, brain, kidney, liver, ovary, pituitary gland	*Low expression* Thyroid, most of brain, Adrenal gland, spleen (human), liver, kidney, heart, intestine, testis (human)

the lack of specific and useful radioligands, because of low expression levels and because of species differences. Even though expression profiles of the different receptors in various organs are known, the evidence for expression of more than one receptor subtype in different cell types is less clear-cut. Thus, it is often uncertain whether expression of adenosine receptor subtypes in the same tissue is due to co-expression on the same cells or coexistence on neighboring cells.

It has, for example, been a matter of debate whether functional adenosine A_3 receptors are present in the brain (Rivkees et al., 2000). Recent unpublished results from our group indicate that adenosine A_3 receptor mRNA is highly expressed in microglia cells, whereas these cells are negative for adenosine A_1 receptors, a subtype which is present in neurons throughout the brain. On the other hand, mouse microglia cells appear to co-express adenosine A_{2A}, A_{2B} and A_3 receptors. With regard to the role of adenosine receptor-mediated signaling and the possible interactions between different signaling pathways, the co-expression of adenosine receptors on the same cells is an important issue in this thesis.

G protein coupling of adenosine receptors

The original classification of adenosine receptors was based on their effects on cAMP levels in different tissues, resulting in the outdated nomenclature of R_i and R_s. Although there are now four known adenosine receptors, and the nomenclature has been changed and refined, the early classification scheme still mirrors the generally accepted profile of adenosine receptor-G protein coupling (see Table 2). Hence, the adenosine A_1 and A_3 receptors mediate a decrease in cAMP, and the two A_2 receptors mediate an increase in cAMP via $G_{i/o}$ and G_s, respectively. A_1 receptors couple to $G_{i1,2,3}$ and G_o (Freissmuth et al., 1991; Akbar et al., 1994; Jockers et al., 1994), while A_3 receptors were shown to couple to $G_{i2,3}$ and, possibly, $G_{q/11}$ (Palmer et al., 1995). There is one report that suggests adenosine A_3 receptor coupling also to G_s in renal epithelial A6 cells (Reshkin et al., 2000), but the evidence for that conclusion was only based on the sensitivity to CTX. Adenosine A_{2A} receptors generally couple to G_s, but a recent study (Kull et al., 2000) showed that A_{2A} receptors in GABAergic striatopallidal neurons are co-expressed with G_{olf} rather than G_s. In addition, immunoprecipitation studies showed that stimulation of A_{2A} receptors activates G_{olf} in rat striatal membranes (Kull et al., 2000). Furthermore, adenosine A_{2A} receptors expressed in COS-7 cells were shown to couple to $G_{15/16}$ (Offermanns and Simon, 1995), but there is no evidence that this interaction occurs *in vivo*. Investigating endogenously expressed adenosine A_{2A} receptors in human endothelial cells, Sexl et al. (1997) cautiously proposed signaling via $G_{12/13}$ proteins without providing direct experimental evidence for this hypothesis. The adenosine A_{2B} receptor, sometimes called the low affinity adenosine receptor (Beukers et al., 2000), also generally couples to G_s, but several studies implicated signaling via $G_{q/11}$ mainly based on A_{2B} receptor-mediated changes in inositolphosphate production and intracellular calcium, which were sensitive to inhibition of PLC blockers, but not to PTX (Yakel et al., 1993; Feoktistov et al., 1994; Linden et al., 1999; Gao et al., 1999). Direct experimental evidence for $G_{q/11}$ activation by A_{2B}, such as immunoprecipitation studies and photolabeling with labeled GTP analogues is, however, not available yet. Recently, coupling of adenosine A_{2B} receptors to $G_{12/13}$ in human endothelial cells was suggested (Feoktistov et al., 2002). However, this remains to be proven directly.

Table 2: G protein coupling of adenosine receptors in systems that both endogenously as well as heterologously express the receptors. [1] – receptor transfected cell system.

Adenosine receptor subtype	G protein	Effects of G protein coupling	Cellular system	References
A_1	$G_{i1/2/3}$	↓ cAMP ↑ IP_3/DAG (PLC); βγ -mediated ↑ Arachidonate (PLA_2)	General, CHO cells[1]	Munshi et al., 1991; Akbar et al., 1994; Freund et al., 1994; Jockers et al., 1994 Freissmuth et al., 1991; Dickenson and Hill, 1998
		↑ choline, DAG (PLD)	DDT_1MF-2	Gerwins and Fredholm, 1995
		↑ K^+ channels	Atrial cells, neurons	Belardinelli and Isenberg, 1983; Trussel and Jackson, 1985
		↓ Q, P, N-type Ca^{2+} channels	Neurons	Dolphin et al., 1986; Scholz and Miller, 1991; Mogul et al., 1993
	G_o			Munshi et al., 1991; Jockers et al., 1994
A_{2A}	G_s	↑ cAMP	General	Olah, 1997
	G_{olf}	↑ cAMP	Striatum, CHO cells[1]	Kull, et al., 2000
	$G_{15/16}$	↑ IP_3	COS-7 cells[1]	Offermanns and Simon, 1995
A_{2B}	G_s	↑ cAMP	General	Pierce et al., 1992
	$G_{q/11}$	↑ IP_3/DAG (PLC)	Xenopus oocytes[1], HMC-1[1], HEK-293[1]	Yakel et al., 1993; Gao et al., 1999; Linden et al., 1999
A_3	$G_{i2,3,\ (o)}$	↓ cAMP	General, CHO cells[1]	Palmer et al., 1995
		↑ IP_3/DAG (PLC) βγ-mediated	General, CHO cells[1]	Ali et al., 1990; Abbracchio et al., 1995
		↑ choline, DAG (PLD)	Rat mast cells	Ali et al., 1996; Parsons et al., 1998
		↑ K^+-ATP channels	Cardiac cells	Tracey et al., 1998;
		↑ Cl^- channels	Human ciliary epithelial cells	Mitchell et al., 1999; Carré et al., 1999
	$G_{q/11}$	↑ IP_3/DAG (PLC)	CHO cells[1]	Palmer et al., 1995

Adenosine receptor signaling - coupling to second messenger pathways

The four adenosine receptors couple to an intricate network of G proteins (see table 2), which enable the endogenous modulator adenosine to communicate with cells in a very complex manner (Fredholm et al., 1996, 2000, 2001a). In early research on intracellular signal transduction signaling pathways were described as linear leading from the receptor

embedded in the cellular membrane via different steps including small signaling molecules called second messengers, protein kinases to gene regulation by transcription factors. Prominent examples are the cAMP-cAMP-dependent protein kinase (PKA) pathway (Daniel et al., 1998), which is regulated by G_i and G_s proteins, and the PLC-inositoltrisphosphate (IP_3) and diacylglycerol (DAG) pathway activated by $\beta\gamma$ subunits and $G_{q/11}$ (Sternweis and Smrcka, 1993; Rebecchi and Pentyala, 2000). Nowadays, the network of intracellular signaling is being revealed in increasingly complicated detail (Bhalla and Iyengar, 1999; Dumont et al., 2001; Neves et al., 2002) and below I will briefly describe those pathways, which are relevant for adenosine receptor signaling dealt with in this thesis.

cAMP/ PKA-pathway

Adenylyl cyclases (AC) comprise a family of transmembrane proteins, catalyzing the formation of cAMP from ATP and exist in nine different isoforms, AC I-IX, which are differentially affected by G_α, $\beta\gamma$ subunits and intracellular calcium (Tang and Hurley, 1998; Hanoune and Defer, 2001). Generally, all ACs are activated by G_s, while G_i proteins inhibit only AC I, V, and VI. $\beta\gamma$ subunits affect ACs both positively (AC II, IV, VII) as well as negatively (AC I). The production of cAMP is furthermore tightly regulated by feedback loops involving protein phosphorylation and calcium, where calcium has positive (AC I, VIII) or negative (AC V, VI) effects (Hanoune and Defer, 2001). cAMP, a freely diffusible molecule, binds to the two regulatory subunits (R) of PKA, which dissociate to release two catalytically active subunits (C) that phosphorylate cytoplasmatic or nuclear targets, such as GPCRs, AC, ion channels or transcription factors. Regulatory and catalytic subunits appear also in different flavors i.e. isoforms and splicing variants that allow for diverse and specific intracellular signaling. For example the regulatory RI isoforms do not, in contrast to RII isoforms, interact with the cAMP-dependent protein kinase (PKA) anchoring proteins AKAP (Daniel et al., 1998) resulting in a different cellular localization and coupling to receptor systems between the two isoforms.

A major target for PKA is called cAMP response element binding protein (CREB), which regulates gene transcription by binding to its consensus site cAMP response element CRE (Mayr and Montminy, 2001). CREB activity is regulated by phosphorylation at Ser 133 resulting in dimerization of this helix-loop-helix protein to allow DNA binding. For full transcriptional activation of CREB, cofactors such as CREB binding protein CBP are necessary. While CREB phosphorylation at Ser 133 requires PKA, phosphorylation and activation of CBP presents possibilities for cross signaling with other kinases, such as the mitogen-activated protein kinases (Grewal et al., 2000).

Receptor-independent activation of AC can be accomplished by the addition of the diterpene forskolin (Seamon and Daly, 1981), and the stable and membrane-permeable analogues of cAMP, such as 8-Br-cAMP, can be used to mimic effects of cAMP.

PLC/ IP_3, DAG pathway

Phospholipases hydrolyze phospholipids leading to the release of small molecules that act as second messengers. In the case of PLCβ, two second messengers are released: water-soluble IP_3 and the lipid-soluble DAG. PLCβ is activated by $G\alpha_{q/11}$ but also by $\beta\gamma$ subunits, e.g. released by PTX-sensitive G proteins activated by adenosine A_1/ A_3 receptors (Dickenson and Hill, 1998). IP_3 diffuses into the cytoplasm, binds to and activates its receptor, which is an ion channel located on the endoplasmatic reticulum. Its

activation leads to a rapid and transient increase in the cytoplasmatic calcium concentration $[Ca^{2+}]_i$. This, in turn, will increase the activity of calcium-dependent protein kinases (PKC) or other calcium binding proteins, such as calmodulin or AC. In addition to an increase in $[Ca^{2+}]_i$, binding of PKC to DAG, which results in the translocation of the kinase to the membrane, is required for full activation of the kinase. Thus, the release of two independent molecules as second messengers has a concerted effect on PKC activation and its localization. The family of PKC consists of three different classes (Mellor and Parker, 1998): classical, novel and atypical PKC (c, n, aPKC), which differ in their modes of regulation (Newton, 1997; Liu and Heckman, 1998): cPKCs (α, βI, βII, γ), nPKCs (δ, ε, η, μ, θ) and aPKCs (ζ, λ, ι). The cPKCs are activated by phosphatidylserine in a Ca^{2+}-dependent manner and they also bind DAG. Phorbolesters can also activate cPKCs and nPKCs: they bind to the C1 domain of cPKCs and nPKCs, which is the binding site for DAG. nPKCs are insensitive to Ca^{2+} but can still be activated by phorbolesters, phosphatidylserine, and DAG, whereas aPKCs respond neither to Ca^{2+} nor to DAG/phorbolesters. Long-term treatment of cells with phorbolesters is – besides its acute activating effects – used to downregulate nPKCs and cPKCs, which expression underlies a negative feedback loop (Chen, 1993).

Signaling via PI3K

Coupling to phosphatidylinositol-3′-kinase (PI3K) via βγ subunits adds another level of complexity to cellular signaling via generation of differentially phosphorylated lipids, such as phosphatidylinositol-(3)-phosphate (PIP), phosphatidylinositol-(3, 4)-bisphosphate (PIP_2) and phosphatidylinositol-(3, 4, 5)-trisphos-phate (PIP_3). The family of PI3Ks is divided into class I_A, I_B, II and III, all consisting of one regulatory and one catalytic subunit (Vanhaesebroeck et al., 1997; Vanhaesebroeck and Waterfield, 1999). Class I_A, I_B and II PI3Ks may be regulated by GPCRs and heterotrimeric G proteins.

All PI3Ks are sensitive to inhibition by wortmannin (nanomolar concentrations) (Arcaro and Wymann, 1993; Woscholski et al., 1994) or LY294002 (micromolar concentrations). Wortmannin was used to elucidate the role of PI3K in mediating insulin actions, cell survival and cell cycle control, inflammatory events and apoptotic signaling.

Phosphatidylinositolphosphates activate various signaling proteins; however, the main pathway is the activation of the protein kinase B (PKB)/ Akt pathway (Vanhaesebroeck and Alessi, 2000). The proto-oncogene PKB/ Akt is a serine threonine kinase related to PKA and PKC. PKB/ Akt is indirectly activated downstream of PI3Ks by phosphorylation through phosphoinositide-dependent kinase-1 (PDK-1) at a threonine residue in the activation loop and a serine residue (Ser-473) at the C-terminal hydrophobic region (Toker, 2000). Catalytically active PKB/ Akt, then in turn phosphorylates several cytoplasmatic proteins, such as glucogen synthase 3 β (GSK-3 β), nuclear factor-κB (NF-κB), the pro-apoptotic Bcl-2 family member BAD, and the ribosomal protein kinase S6 kinase. Thus, besides mediating metabolic effects of insulin, PI3K and PKB/ Akt also provide a survival signal to cells that counteracts apoptosis, mainly by inhibiting the function of BAD.

In summary, these signaling pathways are generally activated by GPCRs and there is substantial documentation in the literature showing that adenosine receptors – according to their G protein coupling – activate the above-mentioned second messenger systems (Fredholm et al., 1996, 2001a; Klinger et al., 2002a). Thus those pathways may be the means by

which adenosine receptors communicate with mitogen-activated protein kinase (MAPK) signaling.

MAPK and the classical MAPK cascade

The MAPK protein family was first discovered as a module in the pheromone-induced mating pathway in the yeast *Saccharomyces cerevisiae* (Herskowitz, 1995) and was later shown to be involved in cell cycle progression, proliferation and differentiation in all organisms including mammals (Widmann et al., 1999). This well-conserved and diverse protein family consists of three main groups, the extracellular signal-regulated protein kinases (ERK), the stress-activated protein kinases (SAPK) p38 and the SAPK c-jun N-terminal kinases (JNK) (Chen et al., 2001). Recently, other kinases have been included into the MAPK family based on structural relationships (Miyata and Nishida, 1999).

MAPKs are proline-directed serine/ threonine kinases, which can be doubly phosphorylated at tyrosine and serine/ threonine residues and, thus, activated by upstream dual-specificity kinases. The common phosphoacceptor motif (Thr-Xaa-Tyr or TXY) of the MAPKK phosphorylation site is located in the activation loop of the MAPK and serves as one criterion for their classification into three main groups: ERK,

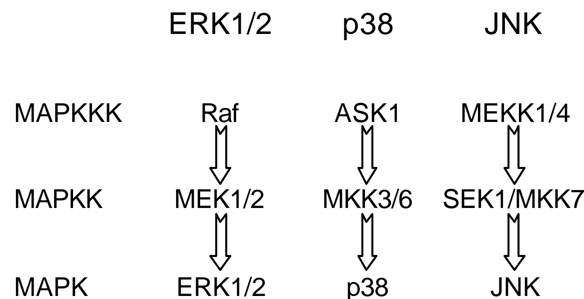

Fig. 3: Schematic drawing of some of the representatives of the three kinase module of the MAPK cascades upstream of ERK1/2, p38 and JNK (MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase).

p38 and JNK carrying T/SEY, TGY and TPY motifs, respectively. The group of mammalian ERK comprises ERK1 (p44 MAPK), ERK 2 (p42 MAPK), ERK 3α (p63 or rat ERK3), ERK 3β (human ERK3), ERK 5 (BMK1), and ERK 7. There are at least four different subforms of p38: p38α, p38β, p38γ, and p38δ. The group of JNK consists of at least 3 isoforms, the JNK1-3 (Widmann et al., 1999; Chen et al., 2001). The increasing number of MAPK gives rise to an enormous signaling network, and we are far from understanding the totality of its impact and complexity in the determination of cellular fate.

The classical MAPK pathway was originally described as a triple protein kinase module which was activated downstream of receptor tyrosine kinases (RTK) e.g. growth factor receptors (Schlessinger, 2000). Ligand binding to RTKs induces receptor dimerization and cross-auto-phosphorylation at a series of tyrosine residues. These P-Tyr sites serve as docking sites for adapter proteins, such as Shc and Grb2, containing src-homology 2 (SH2) domains. Grb2 serves to recruit a guanine nucleotide exchange factor (GEF) for the small monomeric G protein Ras to the RTK. The GEF for Ras is Sos, a mammalian homologue of Son-of-sevenless, discovered because of its involvement in retinal development in *Drosophila melanogaster*. Sos induces the exchange of GDP for GTP at the membrane-anchored Ras, which in turn will activate a serine/ threonine kinase called Raf and recruit it to the membrane. Raf is the first part in the triple kinase module of the MAPK cascade and is therefore also called MAPK kinase kinase (MAPKKK). MAPKKK activates a dual-specific MAPKK by serine/ threonine phosphorylation, which in turn will phosphorylate MAPK (see Fig. 3). MAPKKKs can be grouped into three main families, Raf, the MAPK/ ERK kinase

kinases (MEKK), the mixed lineage kinases (MLK) and the thousand and one kinases (TAOs) (Ono and Han, 2000; Kyriakis and Avruch, 2001).

MAPK phosphorylation and activation of its enzymatic activity are very closely linked events and may therefore be used interchangeably as a measure of protein activity. MAPK activity *per se* is not the final goal of the signaling cascade. Instead, MAPK can phosphorylate a series of different transcription factors, such as Elk-1, MEF2, ATF-2, CREB, ternary complex factor (TCF) and also different cytoplasmatic proteins such as the signal-transducer and activator of transcription (STAT), tyrosine hydroxylase, MAPK activated protein kinase (MAPKAP kinase 2), ribosomal S6 kinase, etc.

GPCR activating MAPK

Not only RTK, but also GPCRs are capable of activating MAPKs using a rather intricate signaling network (Gutkind, 1998a-b, 2000; Lopez-Ilasaca, 1998; Dikic and Blaukat, 1999; Luttrell et al., 1999; Liebmann, 2001; Marinissen and Gutkind, 2001; Belcheva and Coscia, 2002). The first publications linking GPCRs to the MAPK cascade identified G_i-coupled receptors, βγ subunits and the small GTPase Ras as central players in this pathway (Crespo et al., 1994; Faure et al., 1994; Koch et al., 1994a). Later, G_q-coupled receptors (Hawes et al., 1995) as well as receptors coupling to G_z and G_{12} (Belcheva et al., 2000) were suggested to signal to MAPK. The link from the mitogenic effects of G_s-coupled receptors and activation of MAPK (Faure et al., 1994; Frodin et al., 1994; Young et al., 1994; Pan et al., 1995), however, remained unclear (Lamy et al., 1993; Withers et al., 1995; Withers, 1997). In general signaling from GPCRs to ERK1/2 is known in greater detail than signaling to the SAPK p38 or JNK. Depending on the G protein coupling and the cell type or MAPK under investigation, GPCR-activated signaling pathways interact with the MAPK cascades on different levels:

Modulation of ERK1/2 via cAMP

Stimulation of G_s-coupled receptors can have negative and positive effects on ERK1/2 phosphorylation mediated via cAMP (Stork and Schmitt, 2002) (see Fig. 4) and therefore on mitogenesis. In addition, oncogenic mutations of the G_s proteins, so called *gsp*, were discovered (Landis et al., 1989). Transfection of these constitutively active G_s proteins into different cell lines leads to an increased proliferation (Zachary et al., 1990). However, it became clear during the past decade that the final result of cAMP signaling on cell fate is highly cell-specific (Houslay and Kolch, 2000) and one important difference between cells is the presence or absence of the MAPKKK B-Raf. This Ser/ Thr-kinase is - in contrast to Raf-1 - not ubiquitously expressed (Hagemann and Rapp, 1999). B-Raf is for example widely expressed in the brain and in various different cell types: endocrine cells, cells of neural crest origin, endothelial cells, prostate cells, and certain fibroblasts (Stork and Schmitt, 2002, and references therein). The classical downstream target of cAMP is the Ser/ Thr-kinase PKA, which is an important player in MAPK activation. ERK1/2 activation via PKA – in either the presence or absence of B-Raf – may be mediated by activation of Ras (Enserink et al., 2002; Nørum et al., 2002). Another important player in the cAMP-mediated activation of ERK1/2 is the small GTPase Rap1. Rap1, which was originally described as an antagonist of Ras signaling, called K-Ras revertant (Krev-1) (Kitayama et al., 1989), can be activated in a PKA-dependent manner (Quilliam et al., 1991; Vossler et al., 1997). The steps leading from PKA to Rap1 were recently

characterized by Schmitt and Stork (2002) showing that PKA activates Src by phosphorylation at Ser17. Src, in turn, activates the GEF for Rap1, C3G, thereby increasing Rap1 activity. The involvement of Src-like kinases in the PKA-dependent activation of ERK1/2, probably via Rap1 and B-Raf could be confirmed in different cell systems, including brown adipocytes (Fredriksson et al., 2000; Lindquist et al., 2000). PKA may also phosphorylate Rap1 directly; however, the function of this is unclear (Altschuler et al., 1995).

In the case of Rap1 activation the expression of B-Raf is crucial in determining the final outcome for ERK1/2 phosphorylation (and proliferation), because Rap1 will compete with Ras for Raf-1 binding and thereby inhibit the activation of Raf-1 if B-Raf is not present. However, in the presence of B-Raf, Rap1 will activate it and, thus, feed into the triple module of the MAPK cascade leading to ERK1/2 activation (Vossler et al., 1997; York et al., 1998). Similarly, the PKA-mediated activation of Src-like kinases by direct phosphorylation – as first described 1983 (Roth et al., 1983) and recently by Stork and co-workers (Schmitt and Stork, 2002) – may affect ERK1/2 both positively and negatively, depending on the expression of B-Raf (Schmitt and Stork, 2000, 2001).

In addition, another inhibitory pathway from PKA to the MAPK cascade was suggested when a PKA-dependent inhibition of growth factor-mediated ERK1/2 activation was observed: PKA is suggested to inhibit Raf by direct phosphorylation on Ser residues Ser43, 259, and 621 (Cook and McCormick, 1993; Mischak et al., 1996), and later it was shown that Raf is a substrate of PKA – at least *in vitro* (Peraldi et al., 1995). This interaction, however, is not yet fully understood.

Furthermore, PKA-independent effects of

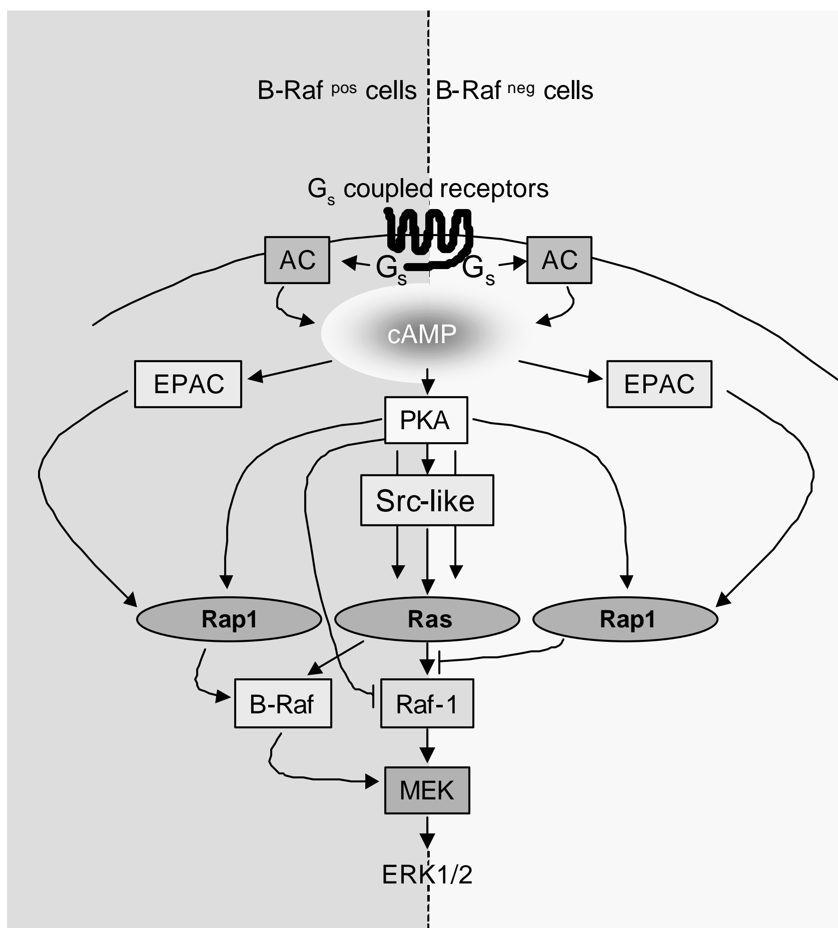

Fig. 4: Modulation of ERK1/2 via G_s-coupled receptors and cAMP in B-Raf expressing (B-Raf^{pos}) and non-expressing (B-Raf^{neg}) cells. (AC, adenylyl cyclase; Epac, exchange factor protein activated by cAMP; PKA, cAMP-dependent protein kinase; Src-like, members of the Src kinase family; Ras, Rap1, small GTPases of the Ras family; B-Raf, Raf-1, representative of the MAPKKK; MEK, MAPK/ERK kinase; ERK1/2, extracellular signal-regulated kinase 1/2; for more details see text).

cAMP on the ERK1/2 activation may be mediated by a recently described guanine nucleotide exchange factor for the small GTPase Rap1 (Kawasaki et al., 1998; de Rooij et al., 1998). This RapGEF or exchange factor protein activated by cAMP (Epac) exists in several isoforms, which show slightly different cAMP-binding characteristics (Kraemer et al., 2001). Epac shows homology to the cAMP-binding domain of the regulatory subunits of PKA, a similarity that was used as a cloning strategy (Kawasaki et al., 1998). Depending on the presence of B-Raf, Epac-induced Rap1 activation may antagonize Ras signaling or activate B-Raf and thereby the ERK1/2 (Stork and Schmitt, 2002). The finding that regulation of Rap1 and ERK1/2 by cAMP can be independent of each other as shown by Enserink et al. (2002) using a novel Epac-specific cAMP analogue, however, challenged this hypothesis and confirmed what was previously surmised (Zwartkruis et al., 1998). In the study by Enserink et al. (2002) cAMP increased ERK1/2 phosphorylation via PKA and Ras but not via Epac/Rap1 and B-Raf, even though both Rap1 and B-Raf were activated. With a similar approach – namely the use of overexpression of RapGAP – Klinger et al. (2002b) confirmed that, even though Rap1 and B-Raf are activated upon adenosine A_{2A} receptor stimulation, they are not necessary for ERK1/2 activation by cAMP and PKA. Instead PKA-dependent activation of ERK1/2 was mediated by Src-like kinases.

Still another pathway that mediated ERK1/2 activation in a cAMP-dependent manner, but independent of PKA, Epac and the Ras-exchange factor Sos was described in melanocytes by Buscà et al. (2000). cAMP was shown to activate Ras, Rap1 and B-Raf; however, only Ras, but not Rap1 mediated the cAMP-dependent stimulation of ERK1/2 via B-Raf.

In summary, cAMP signaling has grown increasingly complicated since its discovery as the first second messenger molecule (Robison et al., 1968) and its communication with the MAPK cascade is strongly dependent on the cell type under investigation.

ERK1/2 activation via PLC

PLCβ can be activated by $G_{\alpha q}$ and by βγ subunits (Crespo et al., 1994; van Biesen et al., 1995) released – theoretically – from any G protein. However, the necessary nanomolar concentrations of βγ subunits to activate PLCβ can be released from $G_{i/o}$ proteins, but usually not from G_q or G_s. This may be due to the involvement of accessory proteins, the fact that $G_{i/o}$ proteins are more abundant in cells, while G_s and G_q proteins show lower expression levels or the fact that the βγ combinations coupled to different α subunits show different activity with regard to effector activation. Thus, a high abundance of the α subunit and/ or the receptor appears to be necessary for the release of sufficient βγ subunits in order to activate effectors (Birnbaumer, 1992).

As described above, PLC activation leads to the mobilization of $[Ca^{2+}]_i$ and the activation of primarily cPKC and nPKC. Starting from this, a rather complex network of interactions lies downstream on the path to ERK1/2 activation (Fig. 5). Both calcium and PKC are necessary for activation of a cytoplasmatic tyrosine kinase, Pyk2 (Dikic et al., 1996), which in concert with Src-like kinases phosphorylates RTK and Shc (Della Rocca et al., 1997). This, in turn, will lead, via Grb2, to the activation of the RasGEF Sos, and then via Ras to Raf-1/MEK/ERK1/2 activation (Blaukat et al., 1999). Direct Ras-independent phosphorylation of Raf-1 by PKC is also possible (Heidecker et al., 1992; Kolch et al., 1993). This, on the other hand, has been suggested to only

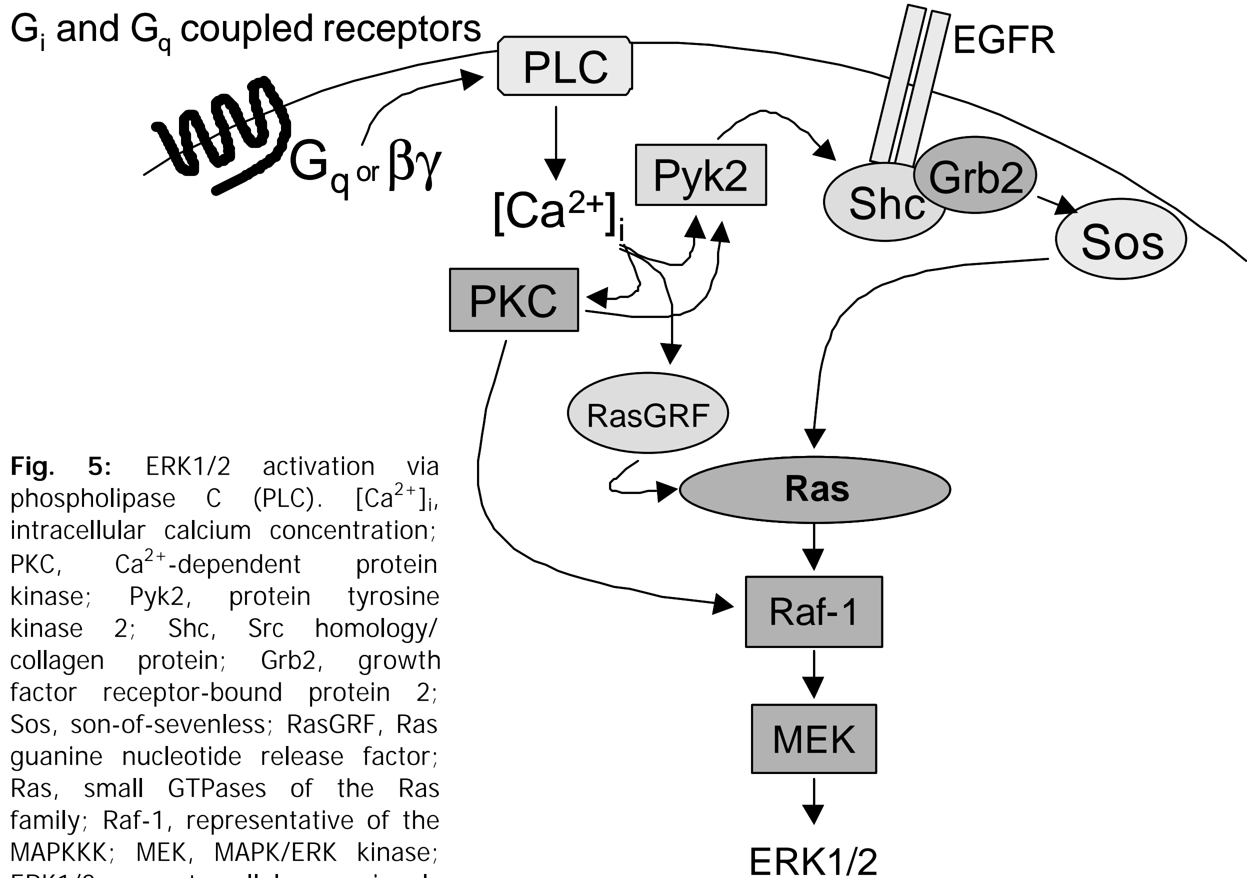

Fig. 5: ERK1/2 activation via phospholipase C (PLC). $[Ca^{2+}]_i$, intracellular calcium concentration; PKC, Ca^{2+}-dependent protein kinase; Pyk2, protein tyrosine kinase 2; Shc, Src homology/ collagen protein; Grb2, growth factor receptor-bound protein 2; Sos, son-of-sevenless; RasGRF, Ras guanine nucleotide release factor; Ras, small GTPases of the Ras family; Raf-1, representative of the MAPKKK; MEK, MAPK/ERK kinase; ERK1/2, extracellular signal-regulated kinase 1/2; for more details see text).

facilitate full activation of Raf by Ras but not to suffice alone for activation of Raf (Burgering and Bos, 1995). The importance of this regulatory mechanism may depend on the PKC isoforms expressed in the cell under investigation and upon receptor levels (Gutkind, 2000). Furthermore, calcium in connection with an as yet unidentified kinase stimulates certain RasGRF that are distinct from Sos (Mattingly and Macara, 1996), which leads to ERK1/2 phosphorylation independent of Pyk2 or PKC. Generally, RasGRF1 rather than Sos is thought to lead to Ras activation downstream of GPCR, while Sos plays a central role in RTK signaling (Farnsworth et al., 1995; Shou et al., 1995; Mattingly and Macara, 1996; Zippel et al., 1996).

ERK1/2 activation via bg subunits independent of PLC

Besides the βγ subunit-mediated activation of PLC, βγ subunits are also capable of activating a series of other upstream regulators of ERK1/2 (Fig. 6). Release of βγ subunits from G_i-coupled receptors leads to the activation of PI3K (Lopez-Ilasaca et al., 1997) and thus to the production of PIP, PIP_2, and PIP_3. Originally, PI3Kγ was identified as the PI3K isoform activated by βγ subunits (Lopez-Ilasaca et al., 1997), and the p110 catalytic subunit of PI3Kγ interacts directly with βγ subunits (Leopoldt et al., 1998), whereas stimulation by βγ subunits also depends on the regulatory p101 subunit associated with PI3Kγ (Stephens et al., 1997). Recently, also PI3Kβ was reported to mediate GPCR signaling (Murga et al.,

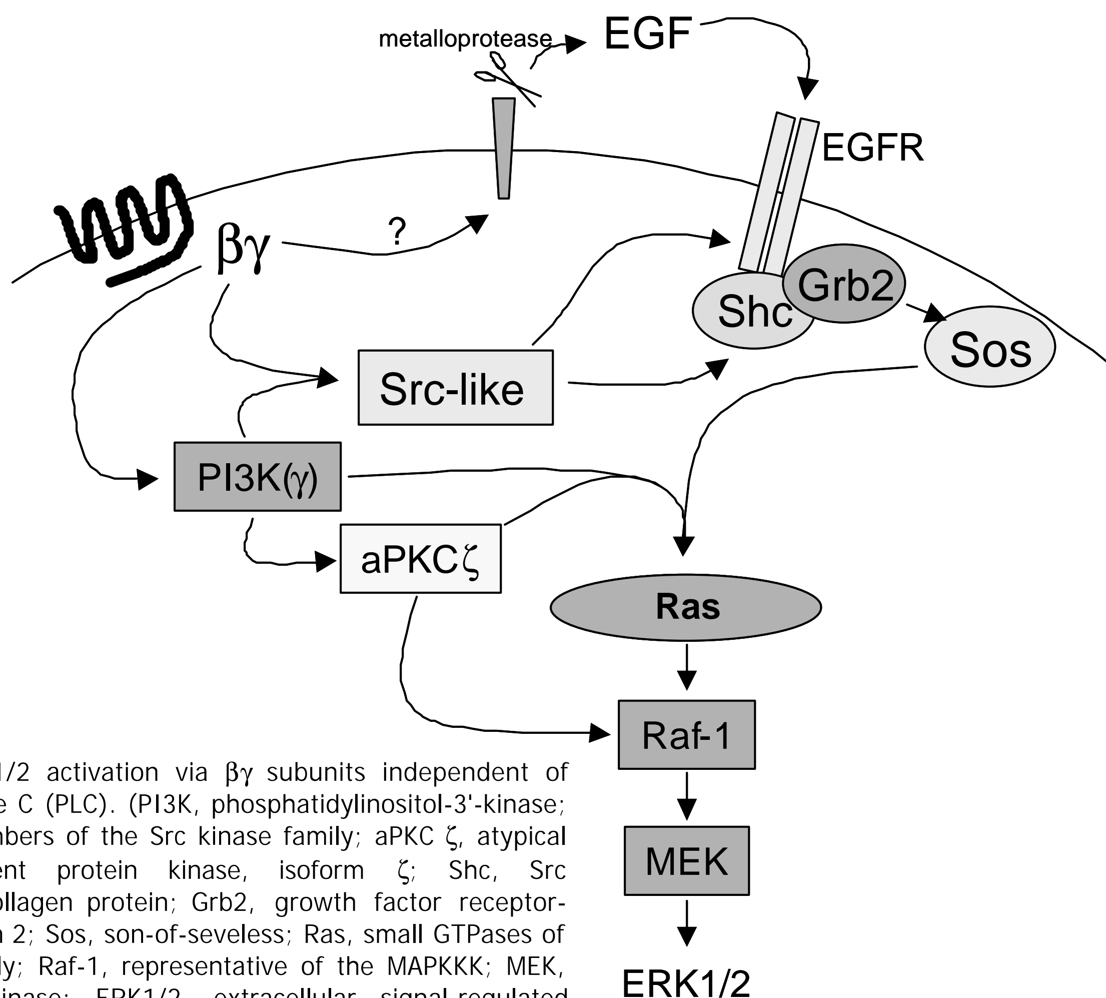

Fig. 6: ERK1/2 activation via βγ subunits independent of phospholipase C (PLC). (PI3K, phosphatidylinositol-3'-kinase; Src-like, members of the Src kinase family; aPKC ζ, atypical Ca^{2+}-dependent protein kinase, isoform ζ; Shc, Src homology/ collagen protein; Grb2, growth factor receptor-bound protein 2; Sos, son-of-seveless; Ras, small GTPases of the Ras family; Raf-1, representative of the MAPKKK; MEK, MAPK/ERK kinase; ERK1/2, extracellular signal-regulated kinase 1/2; for more details see text)

2000). PI3Ks of the class I_A (such as PI3Kβ) and I_B (such as PI3Kγ) contain Ras binding domains (Vanhaesebroeck et al., 1997 and paper IV). Even though most of the data argue in favor of Ras being upstream of PI3K, the opposite has been reported as well (Hu et al., 1995; Vanhaesebroeck et al., 1997), and the truth may lie somewhere in between, i.e. GTP binding of Ras and PI3K activation may be concerted and tightly linked processes (Rubio et al., 1997). In addition, Ras-independent signaling from PI3K to ERK1/2 was reported (Takeda et al., 1999). In the case of Ras being located downstream of PI3K, the way to ERK1/2 is straightforward via Raf-1 and MEK. With PI3K signaling independent of Ras, or Ras being located upstream of PI3K, the diverse phosphatidylinositolphosphates mediate further signaling, for example via Src-like kinases, which can bind PIP_3 with its pleckstrin homology (PH) domain (Pleiman et al., 1994) and translocate to the cell membrane. The Src-like kinases phosphorylate RTK and/or Shc, which will lead to Sos and Ras activation via Grb2 coupling to phospho-Tyr residues (van Biesen et al., 1995; Luttrell et al., 1996). Furthermore, atypical forms of PKC (aPKC), such as the PKCζ, which lack the DAG- and phorbolester-binding C1 domain, are activated by PIP_3 and PIP_2 (Nakanishi et al., 1993), and they were implicated to mediate Ras-dependent (Cussac et al., 1999) or -independent (Takeda et al., 1999) signaling from $G_{i/o}$-coupled receptors to MAPK.

RTK activation upon GPCR stimulation is not always dependent on Src-like kinases. The triple-membrane-passing signal mechanism of EGFR transactivation (Gschwind et al., 2001) can be activated by βγ subunits leading to the recruitment of an unidentified batimastat-sensitive metalloprotease. The proteinase, probably of the ADAM family (Prenzel et al., 1999), cleaves membrane-anchored precursor-EGF extracellularly, which in turn will bind and activate the EGF receptor at the extracellular side of the membrane (Daub et al., 1996). This process is quite remarkable, because it includes a pathway that has both autocrine and paracrine effects on the ERK1/2 activity and even other RTK-mediated processes (Zwick et al., 1999). As this pathway appears to be dependent on βγ-mediated effects it has been mainly described downstream of $G_{i/o}$-coupled receptors but also for G_q-coupled receptors. Except for the β_2-adrenergic receptor transactivation has not been described upon G_s-coupled receptors. Indeed, transactivation via β_2-adrenergic receptors was dependent on PKA-mediated receptor phosphorylation and a switch to G_i coupling but was not directly mediated by G_s-dependent mechanisms (Daaka et al., 1997). RTK transactivation leads via the classical MAPK cascade to a Ras-dependent activation of ERK1/2. Thus, the small GTPase Ras is a central player in the βγ-mediated ERK1/2 activation (Crespo et al., 1994).

Receptor internalization and MAPK activation

GPCRs tend to desensitize upon prolonged agonist stimulation. This process can be divided into several stages: the desensitization of the receptor by phosphorylation reducing its affinity for ligands, the endocytosis of the receptor, removing it from the cell surface, and – in the long term – receptor degradation, reducing the actual receptor number on the cell. The process of receptor phosphorylation turned out to be not only a signal for receptor endocytosis but also a G protein-independent way to signal to MAPK. Different Ser/Thr kinases are involved in this process: the GPCR kinases (GRK), PKC and PKA. A family of proteins, the β-arrestins, bind to the phosphorylated receptor, uncouple it from the G protein and present the basis for extensive scaffolding of signaling proteins. At the same time direct association of β-arrestin with clathrin in clathrin-coated pits will target the receptor- β-arrestin complex for endocytosis. Reaching the endosome, the receptor fate is either recycling and re-exposure at the membrane or degradation in late endosomes. Another task of β-arrestin bound to the receptor is the recruitment of Src-like kinases and/ or diverse MAPK modules containing elements of the Raf-1/MEK/ERK1/2 or the ASK1, MKK4, JNK3 modules (Perry and Lefkowitz, 2002).

Activation of SAPK via GPCR

Besides ERK1/2 GPCRs also activate SAPKs, such as p38 and JNK. A lot of different GPCRs were shown to activate SAPKs; however, the underlying mechanisms are still poorly understood (Gutkind, 2000; Kyriakis and Avruch, 2001; Marinissen and Gutkind, 2001; Pierce et al., 2001). Central players of GPCR-induced SAPK activation are the small GTP binding proteins of the Rho family, especially Rac1 and Cdc42 (Fukuhara et al., 2001). All heterotrimeric G protein families can mediate signaling to p38/ JNK. G_s-coupled receptors activate the SAPK p38 in a cAMP- and PKA-dependent manner (Zhen et al., 1998; Hansen et al., 2000; Zheng et al., 2000; Cao et al., 2001). It was shown that PKA activates the small GTPases of the Rac/Cdc42 group in order to feed into the

p38 MAPK module containing the MKK3/6 (Pomerance et al., 2000).

G_i and G_q-coupled receptors can activate SAPK by means of βγ subunits, PLC, PKC, Pyk2, focal adhesion kinases, diverse GEFs for Rho-family GTPases, etc. (Gutkind, 2000; Marinissen and Gutkind, 2001).

G_{12} proteins do not modulate AC or PLC; instead a series of novel G protein-mediated responses, such as coupling to Rho-like proteins via GEFs, such as Tiam, Dbl, p115 RhoGEF or PDZ RhoGEF, may lead to the activation of SAPK (Prasad et al., 1995; Dhanasekaran and Dermott, 1996; Dhanasekaran et al., 1998; Gutkind, 2000; Sah et al., 2000; Marinissen and Gutkind, 2001).

In parallel to ERK1/2 activation via β-arrestin- and receptor-endocytosis-dependent pathways, JNK signaling is induced by G protein-independent signaling linking the GPCR to a JNK scaffold (Perry and Lefkowitz, 2002). In this case β-arrestin2 complexes the ASK1, MKK4 and JNK3 for example to the angiotensin 1 receptor (McDonald et al., 2000).

Adenosine receptors activating MAPK

Adenosine receptor signaling can both enhance and inhibit proliferation of various different cell types (see table 6). Proliferative effects of GPCRs, including adenosine receptors may be MAPK-dependent or –independent (Withers et al., 1995; Cass et al., 1999). Regarding the network of signaling pathways that may be activated by the four adenosine receptors it is likely that they follow the pattern of other GPCRs and are capable of communicating with diverse MAPK cascades (Gutkind, 1998a, 2000; Marinissen and Gutkind, 2001). Indeed, all of the adenosine receptors either endogenously or heterologously expressed in different cell types were shown to activate at least one subfamily of the MAPK.

Already in 1994, Faure et al. showed by overexpressing βγ-sequestring G_t that the $G_{i/o}$-coupled adenosine A_1 receptor transiently expressed in COS-7 cells mediates ERK1/2 activation via the release of βγ subunits. This βγ-mediated effect may for example be propagated by activation of PLC and the release of calcium (Dickenson and Hill, 1998). However, later, the involvement of PTX-sensitive $G_{i/o}$ proteins, PI3K and MEK could be shown in Chinese hamster ovary (CHO) cells stably transfected with the human adenosine A_1 receptor (Dickenson et al., 1998). Endogenously expressed A_1 receptors in a rat smooth muscle cell line (DDT_1MF-2) couple to ERK1/2 and the SAPK p38 in a PTX-sensitive manner (Robinson and Dickenson, 2001).

ERK1/2 activation via the G_s-coupled adenosine A_{2A} receptor has been studied in primary human endothelial cells (Sexl et al., 1997), stably transfected CHO cells, HEK-293 cells (Seidel et al., 1999; Klinger et al., 2002b), and PC12 cells (Arslan and Fredholm, 2000; Klinger et al., 2002b).

Adenosine exerts an ERK1/2-dependent mitogenic effect on endothelial cells, and the pathway from the A_{2A} receptor to ERK1/2 is Ras and MEK dependent. However, this pathway is independent of G_s, cAMP, PKC, calcium and EGF receptor transactivation. The involvement of G_s was investigated by pretreatment with CTX, which leads to a down regulation of G_s expression. This, however, did not affect the A_{2A} receptor mediated effects on ERK1/2. Thus, the authors (Sexl et al., 1997) proposed coupling of adenosine receptors to $G_{12/13}$ proteins instead of G_s. Similarly, A_{2A} receptor-mediated effects in transfected HEK-293 cells were independent of G_s and cAMP but involved Ras and the RasGEF Sos (Seidel et al., 1999). In contrast, adenosine receptor agonist-induced ERK1/2 phosphorylation in CHO A_{2A} cells could be mimicked by 8-

Br-cAMP and forskolin, and blocked by the PKA inhibitor H89. Receptor stimulation activated the small GTPase Rap1 but not Ras when measured with a GTPase pull-down assay (Seidel et al., 1999). Neither overexpression of RapS17N nor RasS17N, dominant interfering mutants of the two GTPases, could blunt the adenosine A_{2A} receptor-induced ERK1/2 phosphorylation. This was interpreted as an ineffective dominant-negative effect of RapS17N (as was described previously (van den Berghe et al., 1997)). Furthermore, it was shown that CHO A_{2A} cells – in contrast to HEK-293 A_{2A} cells – express B-Raf, which was activated upon A_{2A} receptor stimulation (Seidel et al., 1999). In a follow-up study, which was recently published (Klinger et al., 2002b), the same group beautifully shows the involvement of Src-like kinases in the cAMP/PKA-mediated activation of ERK1/2 in A_{2A} transfected CHO A_{2A}, HEK-293 A_{2A} cells and PC12 cells, which endogenously express A_{2A} receptors. Furthermore it was clarified using expression of Rap1GAP, in order to keep Rap1 in the inactive state, that Rap1 is dispensable for ERK1/2 activation even though Rap1 and B-Raf are activated upon A_{2A} stimulation. Thus, the conclusion of Seidel et al., (1999) suggesting the involvement of Rap1 and B-Raf in the A_{2A} receptor-mediated and PKA-dependent ERK1/2 phosphorylation in CHO cells was hereby corrected (Klinger et al., 2002b). In addition, this pathway appears to be of general importance since similar results were obtained in PC12 and NIH3T3 cells. Unfortunately, they provide no data on involvement of Ras is presented, although recent reports by Nørum et al. (2002) and Enserink et al. (2002) suggest that it is important.

Besides positive effects of adenosine A_{2A} receptor stimulation on ERK1/2 phosphorylation in PC12 cells, the A_{2A} receptor exerts also inhibitory effects on e.g. NGF-induced ERK1/2 phosphorylation, effects which could be mimicked by forskolin (Arslan et al., 1997; Arslan and Fredholm, 2000). This is comparable to the A_{2A} receptor-mediated effects on the G_q-coupled thrombin receptor-induced ERK1/2 phosphorylation in CHO cells (Hirano et al., 1996). Mechanistically, these inhibitory effects of adenosine A_{2A} receptors are not completely clarified yet, because the role of direct phosphorylation of Raf by PKA has not conclusively been proven to be inhibitory. Furthermore, both PC12 and CHO cells contain B-Raf and thus the simple model of Rap1 antagonizing Ras in the absence of B-Raf is not applicable in these cell types. According to Vaillancourt et al. (1994), however, PKA-mediated inhibition of both Raf-1 and B-Raf is the most likely explanation.

The adenosine A_{2B} receptor, a receptor involved in proliferation in many cell types (see table 6) is so far the only one which is known to activate all three families of MAPK: the ERK1/2, p38 and JNK (Feoktistov et al., 1999; Gao et al., 1999; Grant et al., 2001). Studies in human mast cells revealed very different time-courses for the NECA-induced phosphorylation of ERK1/2, p38 and JNK (Feoktistov et al., 1999). Maximal ERK1/2 phosphorylation was reached rapidly and it remained elevated for up to 30 min, while p38 phosphorylation was rapid (maximum at 1 min) but was back to basal levels already after 10 min. JNK phosphorylation, however, reached maximal levels first after 10 min and was back to background after 30 min. The different kinetics of these protein phosphorylations imply that they may be regulated by different upstream events. In the cells studied, adenosine A_{2B} receptor stimulation leads to mobilization of $[Ca^{2+}]_i$ (Feoktistov and Biaggioni, 1995) and, therefore, the receptor may be coupled to $G_{q/11}$ proteins. This is further supported by the fact that

the NECA-induced effects could not be mimicked by forskolin.

With the help of different intracellular enzyme inhibitors it was shown that A_{2B}-induced MAPK activation was involved in IL-8 production in mast cells, as this was inhibited by the MEK inhibitor PD98059 and the p38 inhibitors SB202190 and SB203580 (Feoktistov et al., 1999). The use of tyrosine kinase inhibitors, such as genistein and herbimycin A, which also reduced NECA-induced IL-8 production, does not, however, allow definite conclusions on the downstream signaling elements involved in A_{2B} receptor signaling. Furthermore, PKC signaling was proposed, although not as an absolute requirement but rather as a contribution to A_{2B}-modulated interleukin synthesis (Feoktistov et al., 1999).

In human embryonic kidney cells (HEK-293), which endogenously express A_{2B} receptors, ERK1/2 activation is induced by NECA in a time- and dose-dependent manner showing an EC_{50} value of 0.7 µM, which is rather close to the EC_{50} = 2.7 µM value for cAMP production (Gao et al., 1999). Maximal ERK1/2 phosphorylation levels are reached at 5 min after NECA stimulation. Furthermore it appears that the A_{2B} receptors in HEK-293 cells are – like those in human mast cells – coupled to $G_{q/11}$ because agonist stimulation leads to a rise in $[Ca^{2+}]_i$ (Gao et al., 1999). This assumption is strengthened by the fact that forskolin could not mimic and that H89 could not block NECA-induced ERK1/2 phosphorylation. Instead inhibition of PLC by U73122, of Ras by RasS17N, a dominant-negative form of Ras (Feig and Cooper, 1988), and of MEK by PD98059, inhibits A_{2B}-mediated ERK1/2 phosphorylation. Based on the lack of effect of the PKC inhibitors GF 106203X and Ro-318220 the authors excluded the involvement of PKC as well (Gao et al., 1999). Finally, A_{2B} receptor signaling in HEK293 cells does not involve receptor transactivation or cytoplasmatic tyrosine kinases because both of these should be blocked by 100 µM genistein, which did not affect the NECA-induced ERK1/2 phosphorylation (Gao et al., 1999).

Human retinal endothelial cells express adenosine A_{2B} receptors and NECA stimulation leads to a dose-dependent increase in ERK1/2 phosphorylation with an EC_{50} value in the lower micromolar range (Grant et al., 2001). This effect was insensitive to PKA inhibition by H89 but was blocked by MEK inhibition. This study, however, provides no evidence for the involvement of the G protein involved in ERK1/2 activation. G_s proteins appear to be activated because adenosine A_{2B} receptor stimulation leads to an H89-sensitive increase in CREB phosphorylation. This effect was insensitive to MEK inhibition with PD98059 excluding CREB phosphorylation by an ERK1/2-dependent pathway. Indeed, even though the authors suggest a mechanism involving G_q proteins, they have not excluded G_s and cAMP in the NECA-induced ERK1/2 phosphorylation in human retinal endothelial cells (Grant et al., 2001).

Even though previous studies showed an important role for $G_{q/11}$ in the adenosine A_{2B} receptor-induced ERK1/2 activation – without pointing at an involvement of G_s and cAMP – there may be both cAMP-dependent and –independent pathways for the A_{2B} receptor to signal to ERK1/2. Regardless of the G protein involved – either G_s or $G_{q/11}$ – halfmaximal stimulation of adenosine A_{2B} receptor signaling appears to confirm the previous understanding of this receptor subtype being a "low affinity" receptor (Beukers et al., 2000) with an affinity for adenosine and NECA in the lower micromolar range.

The most recently discovered adenosine receptor, the adenosine A_3 receptor, was first suggested to modulate mitogenesis and the activation of ERK1/2 in human

fetal astrocytes (Neary et al., 1998) based on studies using the unselective adenosine receptor agonist NECA but also the somewhat more A_3-specific IB-MECA. The NECA-induced effects on ERK1/2 phosphorylation could be completely blocked by treatment with bisindol-maleimide (Ro-318220), hinting at an involvement of PKC. The connection between human A_3 receptors expressed in CHO cells and ERK1/2 was described by Graham et al. (2001) reporting a time- and dose-dependent phosphorylation of ERK1/2 with similar EC_{50} values (IB-MECA) in the lower nanomolar range for inhibition of cAMP production and ERK1/2 phosphorylation. As described for $G_{i/o}$ coupled receptors, the effects on ERK1/2 were sensitive to PTX, the tyrosine kinase inhibitor genistein (100 µM) and the two PI3K inhibitors wortmannin and LY294002 as well as the MEK inhibitor PD98059, but insensitive to PKC inhibition. Although all of these intermediates have been suggested to mediate MAPK signaling, it is impossible to draw reliable conclusions from these data: It is still unclear where the different kinases are located relative to each other and there are gaps and uncertainties in the signaling pathway.

Adenosine receptors activating PI3K

Adenosine receptors are – like PI3K – involved in processes such as proliferation, inflammation and apoptosis. Nevertheless, a connection between adenosine receptors and PI3K has hardly been studied. As mentioned above, release of $\beta\gamma$ subunits may directly lead to activation of PI3Kγ or β. Indeed, activation of PI3K was shown in the case of adenosine A_1 receptors (Germack and Dickenson, 2000) and A_3 receptors (Gao et al., 2001) in the smooth muscle cell line DDT_1MF-2 and in rat basophilic leukemia 2H3 mast cells, respectively. Furthermore, signaling from A_3 receptors to ERK1/2 in CHO A_3 cells was sensitive to wortmannin, LY294002, and PTX. Consequently it depends on catalytically active PI3K and was mediated by PTX-sensitive G proteins (Graham et al., 2001).

On the other hand coupling of adenosine A_2 receptors to PI3K has not been reported yet, even though evidence has been provided, that also G_s-coupled receptors and cAMP may affect those phospholipidkinases positively (Cass et al., 1999; Ciullo et al., 2001) as well as negatively (Kim et al., 2001; Wang et al., 2001).

aims

1. To compare adenosine and its metabolite inosine with regard to the ability to activate adenosine receptors in CHO cells expressing each of the four human adenosine receptors.

2. To evaluate the antagonistic effects of xanthines at the human adenosine receptors using a functional assay instead of ligand binding.

3. To compare the ability of adenosine and NECA to induce the phosphorylation of ERK1/2 via each of the human adenosine receptors expressed in CHO cells.

4. To examine if some enzyme inhibitors commonly used in signal transduction research affect adenosine receptors.

5. To describe the intracellular signaling pathway leading from the human adenosine A_3 receptor expressed in CHO cells to ERK1/2.

6. To describe the intracellular signaling pathway leading from the human adenosine A_{2B} receptor expressed in CHO cells to ERK1/2 and p38.

materials & methods

Most of the techniques used in the studies are considered standard methods. For a detailed description of the procedures please see the Materials & Methods section in each individual publication. In the remainder of this section I will briefly comment on some of the techniques and materials used.

Table 3: General techniques, methods and materials used in the studies comprising this thesis.

Method	Paper
Cell culture	I, II, III, IV, V
cAMP measurement	I, II
PAGE/Immunoblotting	I, II, III, IV, V
Radioligand binding	III
$[Ca^{2+}]_i$ measurements	IV, V
Kinase activity assay	IV
Small G protein activity	IV, V
Immunoprecipitation	IV, V
Transfection of cells	IV, V

Chinese hamster ovary cells

In order to compare the pharmacological characteristics of all four human adenosine receptors and to study intracellular signaling via these receptors in cells with identical signaling machinery, the model system of Chinese hamster ovary (CHO) cells expressing the human adenosine A_1, A_{2A}, A_{2B}, and A_3 receptor (CHO A_1, A_{2A}, A_{2B}, A_3 cells, respectively) was created. CHO cells were first described in 1958 as an adherent, fibroblastoid cell line derived from Chinese hamster ovary cells (Puck et al., 1958). Since then it has been used extensively for pharmacological and biochemical characterization of cell surface receptors and downstream signaling events. In 1998 Klotz et al. (1998) characterized the CHO cell lines used in this study in some detail. Untransfected CHO cells were used as internal controls (see paper II, IV and V), because they do not express functional adenosine receptors, as measured by ligand binding and functional studies of adenylyl cyclase activity (Klotz et al., 1998). Even though small amounts of adenosine A_{2B} receptor mRNA could be detected in untransfected CHO cells (Kull et al., 1999a), no functional response could be measured at agonist concentrations resembling physiological or pathophysiological concentrations of the endogenous ligand (paper II, IV, V and Kull et al., 1999a). Thus, the effects induced by adenosine receptor agonists are due to the transfected human receptors. Information on the expression levels of the four different adenosine receptors in CHO cells is summarized in table 4.

With regard to the studies on MAPK signaling it should also be mentioned that CHO cells express the MAPKKK B-Raf as previously reported (Seidel et al., 1999; Ehses et al., 2002) and as shown in Fig. 6. In addition, Ehses et al. (2002) published an overview of different kinases expressed in CHO cells, that may be of interest in MAPK signaling.

NECA

The use of adenosine as an agonist in cell culture or other preparations has some disadvantages: Adenosine has a very short half time (~2 s) in all cell preparations and especially *in vivo* (Moser et al., 1989) because adenosine deaminase is expressed ubiquitously and adenosine transporters are active on every cell. Therefore, the stable agonist NECA (5´-N-

Table. 4: Expression levels of the human adenosine receptors expressed in CHO cells. The clones of the adenosine receptor transfected CHO cells were described previously (Klotz et al., 1998)

Cell type	Receptor expression (fmol/mg protein)	Receptor number/ cell	Comments	References
CHO A_1	~1000	~40,000	Less than in DDT_1MF-2 cells	Gerwins et al., 1990; Klotz et al., 1998; paper II
CHO A_{2A}	247	~20,000	Less than in striato-pallidal neurons	Kull et al., 1999; Klotz et al., 1998
CHO A_{2B}	n.d.	< 200,000	-	Unpublished results, paper II
CHO A_3	807	≤ 40,000	-	Klotz et al., 1998

ethylcarboxamidoadenosine), an adenosine derivative with an N-alkyl carboxyamide substitution at the 5´-carbon atom of the ribose, was used (Raberger et al., 1977). NECA is a full agonist specific for adenosine receptors, but has, however, little selectivity for the different receptor subtypes (Fredholm et al., 2001b). The order of potency of NECA at the different human adenosine receptors ($A_1 \geq A_3 > A_{2A} > A_{2B}$) resembles that of adenosine, at least where cAMP modulation is measured (Klotz et al., 1998 and paper I), and thus NECA is a useful analogue for studying adenosine receptors.

Pull-down assay for small GTP-binding proteins

The mechanistic interactions between small GTPases of the Ras family and their effectors such as Raf and RalGDS for Ras and Rap1, respectively, are not fully understood. The interactions are based on binding of the GTP-bound form of the G protein to the effector with a rather high affinity. This affinity is, indeed, high enough to be taken advantage of for a precipitation assay (Carey and Stork, 2002). A GST fusion protein containing the effector-binding domain of the respective GTPase (Raf-Ras binding domain for Ras; RalGDS-Rap binding domain for Rap1) is used. These glutathione-S-transferase (GST) fusion proteins are overexpressed in bacteria and added to the cell lysate after stimulation and activation of the G protein in question. The complex of GST fusion protein and activated G protein is then precipitated using glutathione-sepharose beads. Finally, the amount of precipitated G protein is determined by immunoblotting using a specific antibody for either Ras or Rap1. The disadvantage of this technique is, however, a limited sensitivity. Successful MAPK signaling may require only 1 % of the whole Ras pool in a cell, and this might not be detectable by the pull-down assay. Hence, positive evidence can be trusted, but not necessarily negative evidence.

ERK1/2 activity

The activity of ERK1/2 has traditionally been determined using the immunoprecipitated kinase for a kinase assay with myelin basic protein (MBP) as the substrate and $[^{32}P]$-γ-ATP for detection of incorporated phosphate. Due to the fact that enzyme activity and enzyme phosphorylation necessarily occur at the same time, the availability of phospho-specific antibodies for the active state of MAPKs revolutionized the field by simplifying the experimental procedures, and it consequently decreased the use of radioactivity. However, the change in enzyme activity still appears to be a more accurate biochemical measure than protein phosphorylation, especially when talking about MAPK *activation*. Thus, in study IV we combined the advantages of a traditional kinase assay with the simpler handling of phospho-specific antibodies: Immunoprecipitated ERK1/2 kinase activ-

ity was measured after a kinase assay by detection of the incorporated phosphate by immunoblotting using a phospho-specific anti-MBP antibody. The results were quantified by densitometry.

Considerations on the specificity of the pharmacological drugs used in this study

Most – if not all – pharmacological tools show a certain degree, frequently a rather considerable degree of non-specificity. Apart from adenosine receptor ligands (adenosine, inosine, NECA, SCH58261, DPCPX, CGS21680, CGS15943, caffeine, theophylline, paraxanthine), drugs acting on intracellular enzymes such as protein kinases (PD98059, Ro-318220, H89, genistein, chelerythrine, PDBu, PP2, PP3), adenylyl cyclase (forskolin, SQ22536), and PI3K (wortmannin, LY294002) were used in this study. Data based on single and high concentrations of a single pharmacological tool do not seem reliable; data become more trustworthy if it is possible to use more than one inhibitor (in the best case structurally unrelated) for the same target over a concentration range covering three or more different concentrations. Furthermore, including negative controls of effects that should not be affected by the drug in question strengthens results (compare CREB phosphorylation in paper V). The fact that a compound has been used in many studies does not protect it from unspecific actions (Alessi, 1997; Davies et al., 2000) and any compound has to be used with care and in combination with suitable control experiments.

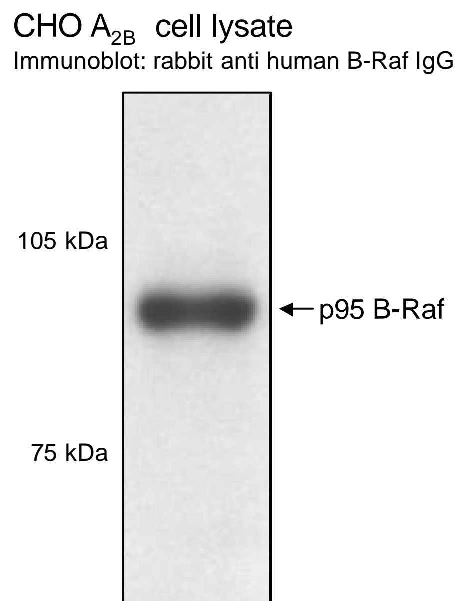

Fig. 7: Expression of the MAPKKK B-Raf (p95 isoform) in CHO A_{2B} cells detected by immunoblotting.

results & discussion

The main focus of this thesis is the comparison of intracellular signaling via the four human adenosine receptors, and finally the detailed description of MAPK signaling via the adenosine A_{2B} and A_3 receptor. As can be seen from the results in paper II, these two receptor subtypes showed the most intriguing profile in ERK1/2 activation, with the A_{2B} receptor mediating the most potent effects of the agonist NECA and the A_3 receptor being most efficient.

Paper I

Adenosine and inosine as ligands at adenosine receptors

In paper I we compared the potency of adenosine and inosine at each of the human adenosine receptors expressed in CHO cells in order to investigate the relative importance of those purines to bind to and activate the human adenosine receptors. We used cAMP as a functional read-out for receptor stimulation, measuring a decrease in forskolin-elevated cAMP levels in the case of $G_{i/o}$-coupled A_1 and A_3 receptors and cAMP increase in the case of the G_s-coupled A_2 receptors.
The potency of adenosine at A_1, A_{2A}, and A_3 receptors to modulate cAMP production was in the nanomolar range (EC_{50} 310 nM (A_1); 730 nM (A_{2A}); 290 nM (A_3)), whereas adenosine A_{2B} receptors were half-maximally activated at 23.5 µM. Inosine, which was completely ineffective at the human adenosine A_{2A} and A_{2B} receptors, mediated half-maximal reduction of forskolin-induced cAMP via the A_1 and A_3 receptors at concentrations about 100 fold higher than adenosine. In addition to the low potency of inosine at A_1 and A_3 receptors, the efficacy (73 and 20% of the adenosine-induced cAMP-decrease, respectively) was low as well, and indeed, effects were hardly measurable in the absence of the adenosine transport inhibitor nitrobenzylthioinosine (NBMPR). This inhibitor was used because extracellular adenosine has a short half-life due to uptake and degradation. Thus, addition of adenosine uptake inhibitors keeps adenosine concentrations in the cell culture medium more constant.
The potency and efficacy of agonists at GPCRs and also at adenosine receptors depend on both agonist concentration and receptor density (Gerwins and Fredholm, 1991; Arslan et al., 1999). The more sparsely the receptors are expressed in a system, the more agonist is required to reach half maximal effects (Kenakin, 1993, 1997). Thus, the human adenosine A_1 and A_{2A} receptors, which show similar or lower expression levels in the CHO cells compared to the *in vivo* situation (see also table 4), are at least partly activated by adenosine during normal physiological conditions. Expression levels of A_{2B} and A_3 receptors in CHO cells are probably higher than those *in vivo*, and therefore it is unclear whether the A_3 receptor is active under normal conditions, although it is obvious that both A_3 and A_{2B} receptors are active when adenosine levels are markedly elevated (for expression levels see table 4). It is likely that inosine – at concentrations that appear during hypoxia and ischemia, for example – can bind and activate adenosine A_1 and A_3 receptors. However, adenosine, which has a much higher affinity to those receptors, is present at approximately the same concentration and thus will compete with inosine for receptor binding. Consequently, inosine has a minimal impact on adenosine receptor signaling *in vivo*.

In addition to agonist potency, we investigated the potency of methylxanthines to antagonize the four human adenosine receptors. While the order of potency at the adenosine A_1, A_{2A}, and A_{2B} receptors for methylxanthines was theophylline paraxanthine > caffeine, none of them exerted a measurable effect at the A_3 receptor at xanthine concentrations encountered in plasma after normal consumption of caffeine-containing products. Thus, caffeine mediates its effects in man by antagonizing mainly adenosine A_1 and A_{2A} receptors – which are activated by basal adenosine levels – while adenosine A_3 receptors are not involved in caffeine effects. Furthermore, adenosine receptor antagonists such as the xanthines enprofylline and theophylline are used in asthma treatment. Thus, our results add to the growing body of data (Salvatore et al., 1993; Forsythe and Ennis, 1999) that the human adenosine A_3 receptor is not the target of anti-asthma therapy based on xanthines. In man, the adenosine A_{2B} receptor, which couples to $G_{q/11}$ in human mast cells (Feoktistov and Biaggioni, 1995; Feoktistov et al., 1999), seems to take over the role proposed for the A_3 receptor in rat and mouse mast cells (Feoktistov and Biaggioni, 1995; Auchampach et al., 1997; Salvatore et al., 2000; Tilley et al., 2000).

Paper II

The human adenosine receptors mediate ERK1/2 phosphorylation

Adenosine and adenosine receptors can mediate proliferative and growth inhibiting effects in a variety of cell types and tissues (see table 6 and Rathbone et al., 1999; Ohana et al., 2001). Even though this was already appreciated more than 15 years ago (Jonzon et al., 1985), the intracellular mechanisms leading to effects on cell growth and differentiation and the roles of the different adenosine receptor subtypes are still unknown and they may or may not depend on the family of MAPKs. Therefore, it appeared interesting to investigate the ability of the human adenosine receptors to induce ERK1/2 phosphorylation and thus ERK1/2 activation. Some of the adenosine receptor subtypes were previously shown to couple to MAPKs (Faure et al., 1994; Sexl et al., 1997; Dickenson et al., 1998; Feoktistov et al., 1999; Seidel et al., 1999; Gao et al., 1999), however the adenosine A_3 receptor had not been studied. Furthermore, no comparative study was available where all the human adenosine receptors had been examined against an identical cellular background.

Adenosine receptor stimulation with NECA and adenosine induced a time- and dose-dependent increase in ERK1/2 phosphorylation (P-ERK1/2), reaching maximal levels at 5 min after agonist stimulation. ERK1/2 phosphorylation was transient and had returned to normal after 30 min in the case of the A_1 and A_{2A} receptor, but it was slightly more prolonged in the case of A_{2B} and A_3 receptors. This may be of significance since it was suggested that the importance of MAPK signaling in different phenotypic changes such as differentiation or cell growth depends on the time the pathway is activated (Qui and Green, 1992; Traverse et al., 1992; Nguyen et al., 1993; Kao et al., 2001). The A_3 receptor mediated the largest increase in P-ERK1/2, followed by the A_1, A_{2A}, and A_{2B} receptors. This may be explained in different ways. Certainly receptor expression levels are one possibility; involvement of different G proteins and signaling pathways another.

NECA was most potent at the adenosine A_{2B} receptor (EC_{50}=19.4 nM), followed by the A_{2A} (EC_{50}=26.4 nM), the A_3 (EC_{50}=65.4 nM) and the A_1 receptor (EC_{50}=115.4 nM). This finding is rather surprising because it does not mirror the results from earlier

studies determining agonist potencies at adenosine receptors, especially at the A_{2B} and A_3 receptors, such as paper I. The order of potency ($A_{2B} \geq A_{2A} > A_3 > A_1$) was very different from that described earlier for NECA (and other agonists) in the same or other cell systems or when a read-out other than protein phosphorylation was chosen. When directly comparing cAMP production and ERK1/2 phosphorylation induced by NECA in CHO A_{2B} cells, an almost 100-fold shift in potency was detected. One explanation for this shift could be that ERK1/2 phosphorylation and cAMP production are independent of each other and that maybe coupling of A_{2B} receptors to $G_{q/11}$ is important in A_{2B}-mediated MAPK signaling.

Paper III

Enzyme inhibitors act directly at adenosine receptors

For the study of signaling pathways downstream of the adenosine A_3 and A_{2B} receptors, which appeared to be the most interesting subjects due to the high efficacy and the high potency of agonists, respectively, we used different pharmacological tools, such as adenylyl cyclase and protein and lipid kinase inhibitors. Results obtained in CHO A_3 cells with genistein and chelerythrine implicated both tyrosine kinases and PKC in the A_3 receptor-mediated ERK1/2 phosphorylation, even though long-term treatment with the phorbolester PDBu did not affect NECA-induced ERK1/2 phosphorylation (Schulte and Fredholm, 2000). These results were explainable with the model presented by Takeda et al. (1999) and it was further confirmed by Graham et al. (2001), who used 100 µM genistein to inhibit NECA-induced ERK1/2 phosphorylation in CHO A_3 cells. Different PKC inhibitors with a broad inhibition spectrum, such as Ro-318220, were, however, ineffective, excluding cPKC, nPKC, and also aPKC (Graham et al., 2001).

When we in parallel investigated the effects of the adenylyl cyclase inhibitor SQ22536 on the NECA- and forskolin-induced CREB phosphorylation in CHO A_{2B} cells, we found that the CREB phosphorylation in the receptor-mediated pathway was more efficiently inhibited than compared to the direct stimulation of AC with forskolin. This was the first hint that SQ22536 may act directly on the receptor and this hypothesis was strengthened by the finding that SQ22536 decreases A_3 receptor-mediated ERK1/2 phosphorylation even though the A_3 receptor is coupled to $G_{i/o}$ proteins, and thus to an inhibition of AC.

Thus, incompatibility of the signaling models in CHO A_3 and A_{2B} cells suggested some unspecific actions of SQ22536, genistein and chelerythrine. Hence, we used radioligand binding ([^{3}H]-DPCPX for

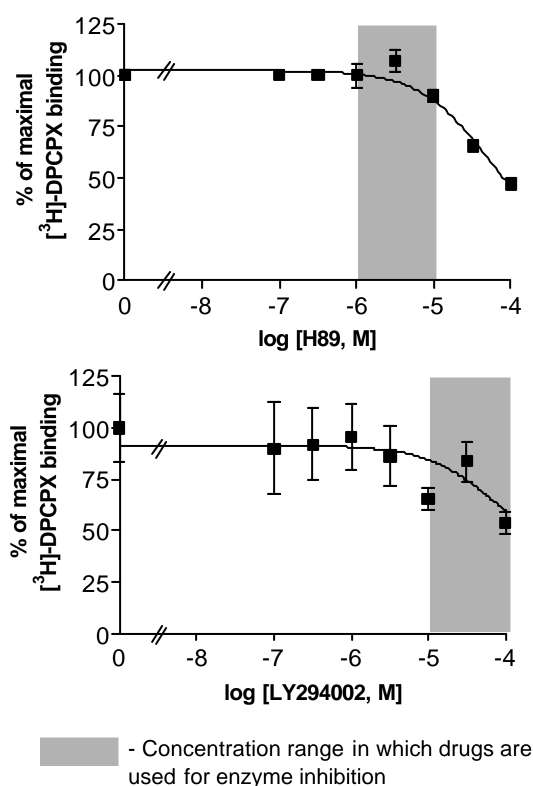

Fig. 8: [^{3}H]-DPCPX binding in rat brain membranes in the presence of increasing concentrations of the PKA-inhibitor H89 or the PI3K inhibitor LY294002.

A_1; [^{3}H]-SCH58261 for A_{2A}) in total rat brain membranes and mouse striatal slices to investigate the ability of these compounds to compete with antagonists for adenosine receptor binding. Indeed, all three compounds – despite varying degree of structural likeness to adenosine – are potent reversible competitive antagonists at the adenosine A_1 and A_{2A} receptors. The K_i values lie roughly in the same concentration range as their IC_{50} values for enzyme inhibition and thus, none of these compounds is suitable for investigation of adenosine receptor signaling. In addition, previous results have to be re-evaluated with regard to the concentrations of inhibitors used and the positive controls included. Our findings may even be relevant for studies not directly examining adenosine receptors, because adenosine receptors are widely expressed, adenosine is present in all cell and tissue preparations at sufficient levels to activate the receptor, and adenosine receptor signaling is known to interact with various other signaling pathways. Given the situation that G_i-coupled adenosine A_1 receptors are present and activated by endogenous adenosine, the addition of SQ22536, genistein or chelerythrine, and, thus, the blockade of A_1 receptors, would result in increased levels of cAMP, which may interfere with the processes under investigation.

Even though we only investigated adenosine A_1 and A_{2A} receptors by radioligand binding, it is very likely that these compounds – and probably all compounds designed to bind the ATP-site of enzymes – interact with the adenosine binding site of all adenosine receptor subtypes. This assumption could be strengthened by results from K.-N. Klotz (personal communication) on [^{3}H]-NECA binding experiments on CHO A_3 cell membranes using increasing concentrations of chelerythrine (K_D 10 µM).

With regard to the concerns raised in paper III about the unspecificity of enzyme inhibitors as adenosine receptor antagonists, we have no reason to believe that H89, LY294002 and wortmannin, as used in paper IV and V, antagonize adenosine receptors. In all of the experiments results are presented that are and are not affected by the drugs and thus serve as an internal control for that problem. Using DPCPX binding, we could verify that H89 and LY294002 do not diminish radioligand binding at A_1 receptors considerably in the concentrations used for enzyme inhibition (unpublished results, see Fig. 8).

Paper IV

The pathway from the adenosine A_3 receptor to ERK1/2

Adenosine A_3 receptor-induced ERK1/2 activation had been suggested to be important for human astrocyte proliferation (Neary et al., 1998). In CHO A_3 cells ERK1/2 phosphorylation induced by NECA is very strong, and the efficient coupling of a GPCR to ERK1/2 in CHO A_3 cells was one of the reasons for studying the intracellular events involved. As described above, adenosine A_3 receptors as $G_{i/o}$ coupled receptors may use a variety of signaling pathways to signal to ERK1/2. With careful use of enzyme inhibitors and molecular biological tools, such as over-expression of βγ-sequestering peptides (βARK-ct) (Koch et al., 1994b) and a dominant-interfering mutant of Ras (RasS17N) we showed that adenosine A_3 receptor signaling to ERK1/2 depends on βγ release from PTX sensitive G proteins, PI3K, Ras and MEK (see also Fig. 9 in paper IV). Even though we could detect a PTX-sensitive influx of calcium upon NECA stimulation, this was not involved in ERK1/2 phosphorylation since Ca^{2+}-chelation by BAPTA had no effect thereon. This, taken together with the fact that

PDBu pretreatment did not affect NECA-induced ERK1/2 phosphorylation, excludes the involvement of cPKC, nPKC and Pyk2 (which appears not to be expressed in CHO cells (Ehses et al., 2002)). In addition Ca^{2+}-insensitive aPKCζ – even though present – is not activated upon NECA stimulation and thus it is unlikely to be involved in ERK1/2 phosphorylation. Independence of PKC is further confirmed by the use of the bisindolmaleiimide Ro-318220, which does not affect NECA-induced ERK1/2 or PKB phosphorylation. Ro-318220 at concentrations above 1 μM abolished the NECA effects, which may, however, be explained by unspecific side-effects, because the IC_{50} value of Ro-318220 for inhibition of aPKC is in the nanomolar range (Wilkinson et al., 1993).

Genistein (30 - 100 μM) inhibits the NECA-induced ERK1/2 phosphorylation in CHO A_3 cells (not shown and Graham et al., 2001). However, this result does not necessarily imply an involvement of tyrosine phosphorylation, as we showed that genistein in this concentration range is an antagonist at adenosine receptors (paper III). The possibility, however, still exists that A_3 receptor-mediated ERK1/2 activation may be evoked by RTK transactivation, which is dependent on PI3K and Ras (Daub et al., 1997), or by activation of cytoplasmatic Src-like kinases. PP2, an inhibitor of Src-like kinases, on the other hand, did not affect NECA-induced ERK1/2 phosphorylation at nanomolar concentrations, which argues against the involvement of this kinase family. With increasing concentrations of both PP2 and its "inactive analogue" PP3, NECA-induced ERK1/2 phosphorylation decreases. This might be explained by unspecific effects (maybe directly at the adenosine receptor, or unrelated cellular targets) or, more importantly, by PP2 and PP3 affecting the epidermal growth factor receptor kinase activity in micromolar concentrations. With regard to the IC_{50} values of PP2 and PP3 at the EGF receptor kinase – 0.5 μM (Hanke et al., 1996) and 2.7 μM (Traxler et al., 1997), respectively – these two inhibitors might affect EGF or RTK signaling and thus argue for the process of receptor transactivation (Daub et al., 1996, 1997; Conway et al., 1999). Except for this evidence, however, we have not investigated receptor trans-activation in more detail.

ERK1/2 phosphorylation via the adenosine A_3 receptor is sensitive to PI3K inhibition by wortmannin and LY294002 (Graham et al., 2001). Indeed, some subtype of PI3K is activated, judging from the indirect read-out of PKB/ Akt phosphorylation. This argues that adenosine A_3 receptor signaling is positively coupled to PI3K and, consequently, that the involvement of PI3K is not primarily reflecting a need for a parallel signal activating the enzyme. We also present evidence for the location of PI3K relative to Ras and the important role of Ras in adenosine A_3 receptor signaling to ERK1/2. Using PI3K inhibitors, a dominant-negative mutant of Ras (RasS17N) and a Ras-activity assay, we conclude that Ras is located downstream of PI3K.

ERK1/2 plays an important role in cell cycle progression and cell growth, thus mediating mitogenic signals. The strong activation of ERK1/2 should therefore lead to an increase in proliferation. In contrast to that, however, CHO A_3 cells react to adenosine A_3 receptor stimulation with IB-MECA or its 2-chloro derivative Cl-IB-MECA with cell cycle arrest and decrease in proliferation (Brambilla et al., 2000). The biphasic effects of adenosine A_3 receptor stimulation on proliferation and cell survival, being stimulatory at low agonist concentrations (Abbracchio et al., 1997b; Yao et al., 1997) and inhibitory at high concentrations (Kohno et al., 1996; Sei et al., 1997), may explain this. Whereas we detect ERK1/2 phosphorylation at 100 nM NECA after 5 min, Brambilla and co-

workers (2000), detect negative effects on cell growth and survival at 30 and 60 µM of IB-MECA and Cl-IB-MECA after 72 h.

Paper V

The pathway from adenosine A_{2B} receptor to ERK1/2 and p38

The adenosine A_{2B} receptor is the adenosine receptor of which we know least regarding e.g. expression in cells, physiological relevance and intracellular signaling (Feoktistov and Biaggioni, 1997). Due to the different potency of NECA to induce cAMP and ERK1/2 phosphorylation in CHO A_{2B} cells (paper II), we investigated the mechanism leading to ERK1/2 but – for a comparison – also to p38 phosphorylation. A differential potency in increasing cAMP levels and activating MAPKs might be explained by differential coupling to heterotrimeric G proteins, such as G_s and $G_{q/11}$. Previous studies have suggested that adenosine A_{2B} receptor-mediated ERK1/2 activation is mediated by $G_{q/11}$-dependent mechanisms, despite the fact that cAMP levels are increased in parallel (Gao et al., 1999; Feoktistov et al., 1999; Grant et al., 2001). In addition, ERK1/2 phosphorylation/ activation and the A_{2B}-mediated increase in cAMP had similar EC_{50} values in the lower micromolar range. Coupling to $G_{q/11}$ is often indirectly measured by determining changes in $[Ca^{2+}]_i$ because $G_{q/11}$ directly couples to PLCβ. However, no mobilization of calcium upon NECA stimulation of CHO A_{2B} or A_{2A} cells could be detected, even though a clear-cut increase could be seen in CHO A_1 and A_3 cells. Thus, the human adenosine A_{2B} receptor does not sufficiently activate $G_{q/11}$ in CHO cells, and $G_{q/11}$-dependent pathways are not responsible for NECA-induced ERK1/2 phosphorylation. Furthermore, a switch from G_s to G_i was proposed being responsible for signaling via G_s-coupled GPCRs, such as the β_2-adrenergic receptor (Daaka et al., 1997). PTX, however, did not affect adenosine A_{2B} receptor-induced effects. In other words, involvement of G_s is more likely, even though cAMP levels are rather low when ERK1/2 and p38 are already half-maximally activated. However, cAMP levels induced by nanomolar concentrations of NECA are also able to increase CREB phosphorylation, with an EC_{50} of 20 nM. Consequently, cAMP levels that are sufficient to mediate half-maximal CREB phosphorylation may also be sufficient for other cAMP-mediated signaling events.

In order to investigate the dependence of MAPK phosphorylation on cAMP we had to rely on mimicking the effect of receptor stimulation rather than blocking cAMP production by inhibitors, such as SQ22536 (see paper III). Therefore, we used forskolin, an activator of AC, and 8-Br-cAMP, a stable and membrane-permeable cAMP analogue. Both of these substances mimicked the effects of adenosine A_{2B} receptor stimulation on CREB, ERK1/2 and p38 phosphorylation, suggesting that all these effects may depend on G_s and cAMP. Preliminary results using interfering RNA (RNAi) (Fire, 1999) designed to decrease the expression of $G_{\alpha s}$, confirm this assumption (not shown, done in cooperation with Dr. Björn Kull).
PKA, as an important element in mediating the downstream effects of cAMP, can be efficiently inhibited with the rather specific blocker H89 that is targeted to occupy the ATP site of the enzyme. As expected, H89 treatment prior to NECA stimulation diminished CREB phosphorylation. p38 phosphorylation was inhibited as well, whereas NECA- and forskolin-induced ERK1/2 phosphorylation were not reduced, rather slightly increased. Thus, CREB and p38 activation are dependent on cAMP and PKA, whereas ERK1/2 activation via A_{2B} receptors requires cAMP but not PKA. This stands in sharp contrast to a recent finding by Klinger et al.

(2002b), who investigated signaling from the human adenosine A_{2A} receptor expressed in CHO cells to ERK1/2. In that case cAMP signaling to ERK1/2 – both that induced via A_{2A} stimulation and that evoked by forskolin/ 8-Br-cAMP – required PKA.

If the adenosine A_{2B} receptor-induced ERK1/2 phosphorylation does not require PKA, it may depend on the GEF for Rap1, Epac, which is directly activated by cAMP (Kawasaki et al., 1998; de Rooij et al., 1998). Even though the importance of this signaling pathway for ERK1/2 activation has recently been questioned (Zwartkruis et al., 1998; Buscà et al., 2000; Enserink et al., 2002), other reports suggest a positive connection between Epac and/or Rap1 and the ERK1/2 (Vossler et al., 1997; Iacovelli et al., 2001; Stork and Schmitt, 2002). However, stimulation of CHO A_{2B} cells with the Epac-specific cAMP analogue 8CPT-2Me-cAMP (Enserink et al., 2002) did not affect basal ERK1/2 phosphorylation even though Rap1 was activated. This indicates that NECA via cAMP activates Rap1, which on the other side may not be sufficient for NECA-mediated ERK1/2 phosphorylation.

PI3K is activated both by NECA and forskolin, indirectly assessed by the detection of PKB/ Akt phosphorylation. Since Rap1 activation is sensitive to LY294002 and wortmannin, two structurally unrelated PI3K inhibitors, PI3K is located upstream of Rap1 and may interact directly with this Ras-family member via its Ras-binding domain (Vanhaesebroeck et al., 1997; Campbell et al., 1998). A very recent report (Mei et al., 2002) implicates Epac/ Rap1 and PKA in concerted modulation of PI3K, a model locating PI3K downstream of Rap1. Cass et al. (1999) proposed cAMP-dependent but PKA-independent activation of PI3K in rat thyroid cells, which agrees with our

Table 5: Summary of adenosine receptor signaling pathways to ERK1/2 and p38 in CHO cells including previously published results as well as unpublished data from this thesis. [1]Genistein was shown to antagonize DPCPX-binding to rat A_1 receptors with a K_i=2.6 µM (paper III). [2]Rap1 and B-Raf were later shown to be dispensable for the A_{2A}-mediated ERK1/2 activation (Klinger et al., 2002b). Note: Signaling pathways do not describe complete chains of events.

Receptor subtype	MAPK subtype	Signaling pathway (in order of events)	Signaling independent of	Agonists used in the studies	Reference
A_1	ERK1/2	$G_{i/o}$, $\beta\gamma$, Tyr kinase[1](?), PI3K, MEK1	PKC	CPA, NECA (EC_{50}=115.4 nM), adenosine	Dickenson et al., 1998; Faure et al., 1994; paper II
A_{2A}	ERK1/2	G_s, cAMP, PKA, Rap1[2], B-Raf[2], MEK1	$G_{i/o}$, Ras	CGS21680, NECA (EC_{50}=26.4 nM), adenosine	Seidel et al., 1999; paper II
	ERK1/2	$G_{\alpha s}$, cAMP, PKA, Src, Ras	$\beta\gamma$, Rap1, receptor internalization, transactivation	CGS21680	Klinger et al., 2002
	ERK1/2	$G_{\alpha s}$, cAMP	PKA	NECA	Schulte and Fredholm, unpublished results
A_{2B}	ERK12	G_s, cAMP, PI3K, EGFR transactivation, MEK1	PKA, (Rap1), G_q, G_i	NECA (EC_{50}=19.4 nM), adenosine	Schulte and Fredholm, paper II, V
	p38	G_s, cAMP, PKA	$G_{i/o}$, PI3K	NECA (EC_{50}= 18.5 nM), adenosine	
A_3	ERK1/2	$G_{i/o}$, $\beta\gamma$, PI3K, Ras, MEK1	c, n, aPKC, receptor internalization	IB-MECA, NECA (EC_{50}=65.4 nM), adenosine	Graham, et al., 2001; Trincavelli et al., 2002; paper, II, IV

model (see Fig. 9 in paper V). In contrast to signaling in CHO A_{2B} cells, however, PI3K activity in thyroid cells did not lead to an increased phosphorylation of ERK1/2. Unfortunately, we have no evidence for the mechanism by which cAMP and PI3K are connected; nor do we know how PI3K activates Rap1. Ras is not activated by either NECA or forskolin in CHO A_{2B} cells, at least when measured with the Ras-pull down assay.

Furthermore, addition of the EGFR inhibitor AG1478 completely blocks the NECA-induced ERK1/2 phosphorylation, without affecting CREB phosphorylation, thus indicating that adenosine A_{2B} receptor signaling to ERK1/2 involves RTK transactivation.

Recent reports showing a cAMP-dependent activation of ERK1/2 propose an important role for PKA, Src-like kinases and Ras (Enserink et al., 2002; Klinger et al., 2002b) or Rap1 (Stork and Schmitt, 2002), rather than Epac and Rap1 or the mechanisms we propose. These models, however, are not applicable in the case of human A_{2B} receptors in CHO cells, because they all are PKA-dependent.

In contrast to ERK1/2 both CREB and p38 activation is mediated via cAMP and PKA as they are sensitive to H89. The connection of G_s-coupled GPCR with the SAPK p38 has been previously reported to be cAMP and PKA-dependent (Zhen et al., 1998; Hansen et al., 2000; Zheng et al., 2000; Cao et al., 2001). However, the remaining steps in the signaling cascade are rather unclear. Downstream of PKA, small GTPases of the Rho family, such as Cdc42 and Rac (Pomerance et al., 2000) may activate a p38 kinase kinase. We have not, however, investigated this signaling pathway in detail.

Another surprising finding, which was described by others without further comment (Iacovelli et al., 2001), was the effect of H89 on the NECA-induced activation of PKB, Rap1, and ERK1/2. Instead of seeing blocking of those effects, we regularly observed a slight increase in the NECA-induced response in the presence of 10 µM H89. This cannot be explained by the removal of an inhibitory input of PKA on the level of Raf (Cook and McCormick, 1993; Peraldi et al., 1995; D'Angelo et al., 1997), because a reduced Raf inhibition would not result in an increase in the NECA-induced Rap1 or PKB activation, which are located upstream of Raf. Hence, the inhibitory tonus of PKA has to target cAMP signaling somewhere upstream of PI3K, maybe even at the G_s protein, the AC or the receptor itself. However, we could see this increase upon 10 µM H89 treatment also in forskolin-treated cells and thus, steps between the AC and PI3K are more likely to be responsible for this observation.

general discussion

Adenosine receptors are involved in a plethora of modulatory functions affecting almost all cell types. However, pharmacologically it is difficult to distinguish between adenosine receptor subtypes, especially when addressing the involvement of the adenosine A_{2B} and A_3 receptor (Fredholm et al., 2001a). Recently, genetically modified animals have been generated lacking one of each of the adenosine receptor subtypes (A_1: (Johansson et al., 2001); A_{2A}: (Ledent et al., 1997); A_{2B}: (Marquardt, 2002); A_3: (Salvatore et al., 2000)), which will help us to understand the physiological role of adenosine receptors in intact animals. The lack of adenosine receptors in cells from knockout animals may enable us to study intracellular signaling events downstream of single adenosine receptor subtypes in the future. Primary cells isolated from genetically modified animals can provide valuable models for studies on the functional role of adenosine receptor-induced MAPK signaling and the signaling pathways involved.

Another way to approach the problem of understanding adenosine receptor function is investigation of intracellular signaling pathways in recombinant systems and this approach is used in this study. The simplicity of recombinant cell expression systems makes biochemical analysis of intracellular signaling events and intervention with molecular biological tools, such as dominant-interfering mutants, possible. The role of major signaling pathways in certain physiological functions is known and may allow extrapolation to the physiological role of adenosine receptor signaling. Starting from there it will be possible to combine the results and techniques in more complex systems such as organotypic cell cultures or organ preparations in order to understand the whole scenario ranging from the changes in extracellular adenosine, to receptor stimulation, diverse intracellular signaling pathways leading to changes in the cellular phenotype and, thus, to the final physiological response to adenosine.

The model of human adenosine receptors expressed in CHO cells was designed for a biochemical and pharmacological characterization of single adenosine receptor subtypes and the pathways leading from adenosine receptors to MAPKs have been a subject of intense investigations (compare table 5). Results from such recombinant cell systems cannot be extrapolated directly to cell types and organs that endogenously express these receptors. However, a combination of these results with what is known about MAPKs and their physiological role, and previous studies on adenosine receptors in various setups offers reasonable grounds for speculations and the formulation of testable hypotheses.

The fact that adenosine modulates cell growth and that adenosine receptors are involved in mitogenic signaling (see table 6) via MAPK suggests that adenosine, which is constantly present to stimulate its receptors, is important for cellular maintenance under normal physiological conditions. The role of the different adenosine receptor subtypes, G protein coupling and the possible interaction of adenosine receptor subtype-specific signaling is necessarily the focus of future experiments.

The literature describes different phenomena where adenosine receptor stimulation and MAPK activation appear in

parallel. To date, however, the causal relationship, if any, remains unproven. For example, rising adenosine levels after hypoxia or ischemia protect cells in an adenosine receptor-dependent manner (Fredholm, 1997; Johansson et al., 2001) and activate MAPKs (Bogoyevitch et al., 1996; Haq et al., 1998; Sugden and Clerk, 1998). Furthermore, MAPKs are implicated in tissue protective effects upon oxygen depletion. Indeed, an important relationship between adenosine receptors and MAPKs was recently reported in perfused rabbit heart, where adenosine is able to activate ERK1/2, p38 and JNK (Haq et al., 1998). However, it is not clear if adenosine receptor-mediated MAPK activation is necessary and/ or sufficient for the protective effects. Adenosine and adenosine receptors as well as MAPK were also implicated in the phenomenon of ischemic preconditioning (Miura and Tsuchida, 1999; Cohen et al., 2000), however, also here a clear causal relationship between adenosine receptor stimulation, MAPK activation and preconditioning remains to be shown. Preliminary results using a model of remote ischemic preconditioning in hearts of mice that lack the adenosine A_1 receptor suggest the involvement of adenosine receptor-dependent phosphorylation of p38 and ERK1/2 in protective effects (Schulte et al., unpublished data).

Results from experiments on the cellular level reveal a functional relevance of adenosine receptor-mediated MAPK activation. Proliferation of endothelial cells, for example, is increased by adenosine A_{2A} receptor stimulation (Sexl et al., 1995) in an ERK1/2-dependent manner (Sexl et al., 1997), thus suggesting that this pathway may have a role in angiogenesis. The adenosine A_{2B} receptor-mediated activation of p38 and ERK1/2, in addition, was shown to be involved in IL-8 secretion in human mast cells, thus resulting in the degranulation of mast cells (Feoktistov et al., 1999). Furthermore, adenosine A_{2B} receptors were suggested to participate in DCC (for deleted in colorectal cancer) and Netrin-1 signaling, suggesting a role for adenosine A_{2B} receptors in early development of the nervous system and axon outgrowth of dorsal spinal cord explants (Corset et al., 2000); phenomena that might be regulated by MAPKs. The involvement of A_{2B} receptors in axon guidance, however, was recently questioned (Stein et al., 2001).

Previously observed effects on MAPKs mediated by A_{2B} receptors – the so-called low affinity adenosine receptor – were induced at ligand concentrations resembling pathophysiological levels of adenosine. If, however, the finding from paper II regarding the potency of agonists to induce ERK1/2, p38 (and CREB) phosphorylation mirrors physiological reality, at least in cells that express adenosine A_{2B} receptors in abundance, the receptors may transfer signals both under normal physiological conditions as well as under conditions where adenosine levels are increased. The phenomenon of adenosine A_{2B} receptors mediating MAPK activation with a high potency could indeed be reproduced in skeletal muscle cells (personal communication with Dr. Martin Hohenegger, Vienna), thus indicating that this finding is not an artefact of human adenosine receptor-expression in CHO cells. Consequently, there may in fact be cells that express adenosine A_{2B} receptors in sufficient numbers to transfer mitogenic signals at physiological adenosine levels.

Moreover, coupling of adenosine A_{2B} receptors to PI3K and the PKB/ Akt pathway via cAMP (paper V) presents a novel signaling pathway for G_s-coupled receptors that has not been described previously. Especially for G_s-coupled receptors activation of PI3K may present a possible mitogenic pathway, which can be

independent of MAPKs. Furthermore, this connection may imply a role of adenosine A_{2B} receptors in inflammation, apoptosis and insulin signaling.

When investigating adenosine receptor signaling in CHO A_3 cells, we discovered coupling to the PI3K-PKB/ Akt signaling pathway (paper IV). In fact similar results were recently obtained showing activation of PKB/ Akt in rat basophilic leukemia cells (Gao et al., 2001) and using PI3K inhibitors in CHO A_3 cells (Graham et al., 2001). This signaling pathway mediates mitogenic effects resulting in increased DNA synthesis and cell proliferation. Furthermore PKB/ Akt is known to protect from apoptosis by direct phosphorylation and inhibition of the Bcl-2 family member BAD. It is intriguing that the A_3 receptor, which had repeatedly been shown to modulate apoptosis (Abbracchio et al., 1997a; Jacobson, 1998; Gao et al., 2001), activates the PI3K-PKB/ Akt pathway, which was suggested to act as an anti-apoptotic survival signal (Khwaja, 1999). The discovery of these intracellular events may represent an explanation for mitogenic and anti-apoptotic effects of adenosine A_3 receptor activation.

Adenosine A_3 receptor-induced ERK1/2 activation is involved in the regulation of the A_3 receptor itself via a feedback loop (Trincavelli et al., 2002). It was shown that NECA induced an internalization-independent activation of ERK1/2, which led to the phosphorylation and translocation of G protein-coupled receptor kinase 2 (GRK2) to the membrane and to adenosine A_3 receptor phosphorylation. Thus, ERK1/2 activation either by adenosine receptors or by other stimuli may lead to a homologous or heterologous desensitization of adenosine receptors. *In vivo*, this can have profound consequences for adenosine receptor

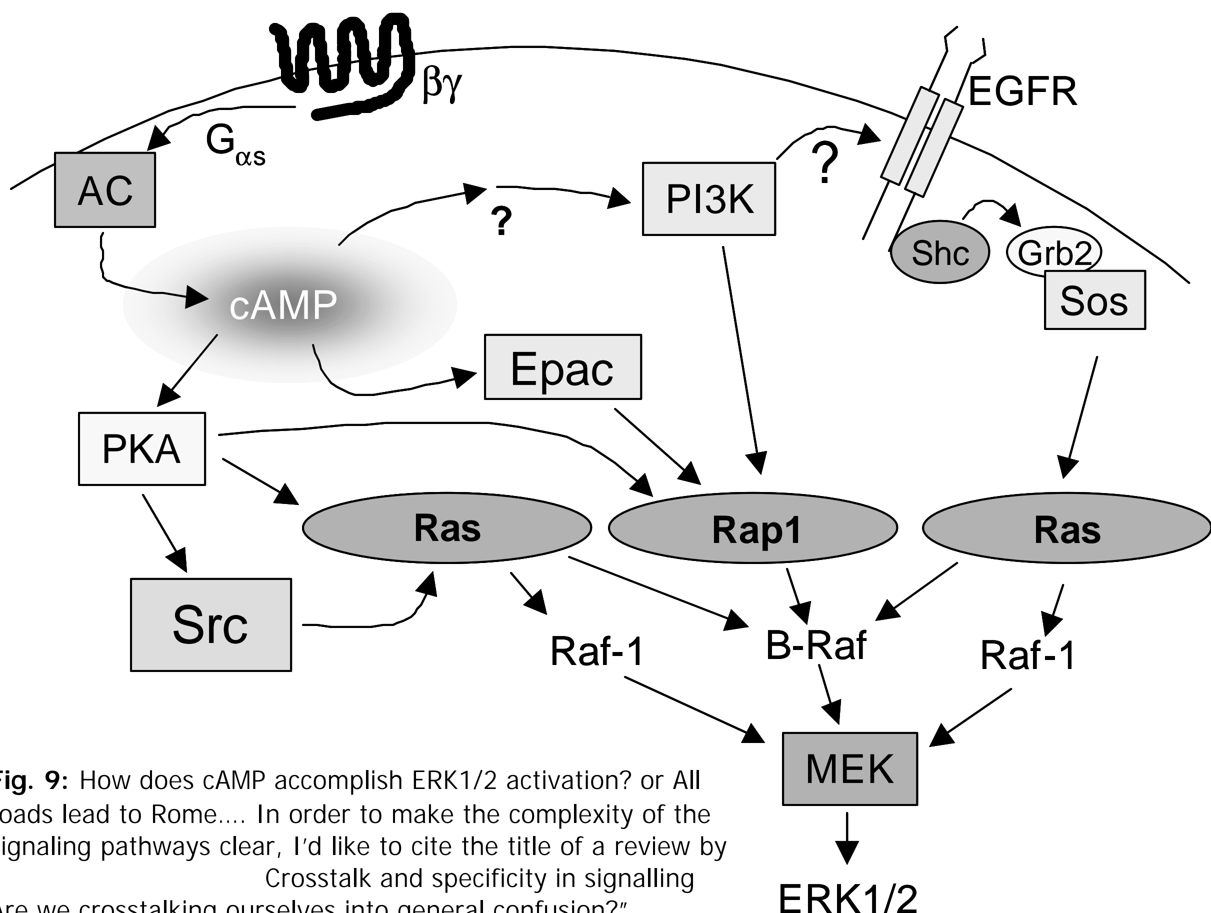

Fig. 9: How does cAMP accomplish ERK1/2 activation? or All roads lead to Rome.... In order to make the complexity of the signaling pathways clear, I'd like to cite the title of a review by Crosstalk and specificity in signalling Are we crosstalking ourselves into general confusion?"

signaling after periods of increased extracellular adenosine concentration that result in a rapid decrease in the receptor density on the cell surface. It is not clear, however, which adenosine receptors are regulated by MAPKs, except for the A_3 receptor. It was shown, on the other hand, that different adenosine receptors desensitize with different kinetics, partly depending on the existence of phosphorylation sites for GRK2 (Ferguson et al., 2000).

Paper V is, indeed, the first study to show that adenosine A_{2B} receptors can increase ERK1/2 and p38 phosphorylation via cAMP-dependent pathways and not via $G_{q/11}$ signaling. An important question arises: How is the adenosine A_{2B} receptor-induced and $G_{q/11}$-dependent activation of MAPKs (Feoktistov et al., 1999; Gao et al., 1999) affected by the parallel increase in cAMP? According to Hirano et al. (1996), $G_{q/11}$-coupled receptor signaling to ERK1/2 is decreased in the presence of adenosine receptor-dependent cAMP signaling, probably via an inhibition of Raf by PKA. It remains, however, to be shown that one receptor can activate two pathways that at least partially antagonize each other.

Furthermore, paper V implicates a role for EGFR in cAMP signaling. The mode of transactivation, however, was not characterized in detail and neither was the location of the EGFR relative to PI3K. But there is evidence the signaling pathway leads from G_s – cAMP – ? – PI3K – ? –

Table 6a: Adenosine receptor-mediated effects on mitogenesis and possible receptor subtypes and signaling pathways involved. Abbreviations for table 6a-d: APNEA, N^6-2-(4-aminophenyl)ethyladenosine; EHNA, erythro-9-[2-hydroxy-3-nonyl]adenine HCl; CCPA, 2-chloro-N(6)-cyclopentyladenosine; CHA, cyclohexyladenosine; 2-Cl-Ado, 2-chloro-adenosine; CPA, N 6-cyclopentyladenosine; DPMA, N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)]ethyladenosine; MECA, 5'-N-methylcarbamoyladenosine; (R/L)-PIA, R(-)N^6-(2-phenyl-isopropyl)adenosine.

Adenosine receptor subtype	Effect on mitogenesis or differentiation	Proposed signaling pathway	Cellular system	Agonist used	Reference
A_1	↑ DNA synthesis	↓ cAMP	Rat arterial smooth muscle cells	L-PIA	Jonzon and Fredholm, 1985
	↑ DNA synthesis	n.d.	Human epidermoid carcinoma cells	Adenosine, R-PIA	Tey et al., 1994
	↑ proliferation	↓ cAMP	Human colonic adenocarcinoma cells	Low concentration NECA	Lelièvre, et al., 1998
	↑ proliferation, DNA synthesis	G_i	Mouse osteoblast-like cells	Adenosine	Shimegi, 1998
	↓ DNA synthesis and proliferation	G_i-mediated decrease in TSH-induced cAMP production	Rat thyroid epithelial cells	L-PIA	Sho et al., 1999
	↓ proliferation, ↑ differentiation	n.d.	Preadipocyte cells (human A_1 receptor transfected)	Receptor expression	Tatsis-Kotsidis and Erlanger, 1999
	↓ proliferation, ↑ cardiac hypoplasia	n.d.	Heart, cardiac cells	CPA	Zhao and Rivkees, 2001
	↓ proliferation	n.d.	Rat pituitary endocrine cells	CCPA, adenosine	Rees et al., 2002

Table 6b: Adenosine receptor-mediated effects on mitogenesis and possible receptor subtypes and signaling pathways involved.

Adenosine receptor subtype	Effect on mitogenesis or differentiation	Proposed signaling pathway	Cellular system	Agonist used	Reference
A_2 subtype not specified	↑ DNA synthesis, cell division	↑ cAMP	Mouse fibroblasts (Swiss 3T3) cells	NECA	Rozengurt, 1982
	↑ proliferation	n.d.	Chick astrocytes	Adenosine	Christjanson et al., 1983
	↓ DNA synthesis	↑ cAMP	Rat arterial smooth muscle cells	NECA	Jonzon et al., 1985
	dedifferentiation	↑ cAMP (?)	Newt iris epithelial cells	Adenosine	Torres et al., 1988
	differentiation	↑ cAMP	Mouse erythroleukemia cells	PIA	Sherman et al., 1988
	↑ DNA synthesis	n.d.	Chick astrocytes	Adenosine	Rathbone et al., 1992
	↓ proliferation	n.d.	Bovine retinal microvascular pericytes	Adenosine	Jackson and Carlson, 1992
	↓ DNA synthesis	n.d.	Human epidermoid carcinoma cells	Adenosine, NECA	Tey et al., 1994
	↓ proliferation, DNA synthesis	n.d.	Rat smooth muscle cell	Adenosine	Dubey et al., 1996
	↑ proliferation	Decreased cAMP	Human colonic adenocarcinoma cells	High concentration NECA	Lelièvre, et al., 1998
A_{2A}	↑ DNA synthesis	n.d.	Human umbilical vein endothelial cell	NECA, CGS21680	Sexl et al., 1995
	↑ DNA synthesis	cAMP, PKA	Mouse mammary epithelial cells	Adenosine, CGS21680, NECA	Yuh and Sheffield, 1998
	↑ DNA synthesis	Ras, MEK, ERK1/2 independent of G_s, cAMP, PKA	Human endothelial cells	CGS21680	Sexl et al., 1997

EGFR – ? – MEK – ERK1/2 (Fig. 9).

These results have more general significance from a signaling perspective, since they not only clarify adenosine A_{2B} receptor signaling but also give more general perspectives for signaling via G_s-coupled receptors.

MAPK signaling affects cell cycle progression and thus the transition from the resting state, G_0 to the G_1 and S-phase by upregulation of cyclins, especially cyclin D_1, downregulation of a cyclin-dependent kinase inhibitor $p27^{Kip1}$, and ultimately the phosphorylation of the retinoblastoma protein (Jones and Kazlauskas, 2000, 2001). It appears that two major signaling pathways are responsible for the growth factor-induced transition through G_1 phase into the S phase: The ERK1/2 and the PI3K-PKB/ Akt pathway. The ERK1/2 pathway seems to be important for the transition from G_0 to G_1 resulting in the induction of an early G_{1E} phase, whereas the PI3K-PKB/ Akt pathway plays a role in the second wave of signaling inducing a late G_{1L} phase, which finally leads to DNA

Table 6c: Adenosine receptor-mediated effects on mitogenesis and possible receptor subtypes and signaling pathways involved.

Adenosine receptor subtype	Effect on mitogenesis or differentiation	Proposed signaling pathway	Cellular system	Agonist used	Reference
A_{2B}	↓ DNA synthesis, proliferation	n.d.	Human aortic smooth muscle cells	Adenosine, 2-Cl-Ado, CGS21680, NECA, MECA	Dubey et al., 1998
	↓ DNA synthesis, proliferation	n.d.	Rat aortic smooth muscle cells	2-Cl-Ado, adenosine, CGS21680, CPA	Dubey et al., 1996; Dubey et al., 1998
	↓ M-CSF-induced proliferation, cell cycle arrest	cAMP, H89 but not ERK1/2, $p27^{kip-1}$, CDK	Mouse bone marrow-derived macrophages	NECA	Xaus et al., 1999
	↓ DNA synthesis, proliferation	Inhibition of ERK1/2 (?)	Rat vascular smooth muscle cells	2-Cl-Ado, NECA, MECA, CGS21680, CPA	Dubey et al., 2000
	↓ DNA synthesis	↑ cAMP	Rat cardiac fibroblasts	Adenosine, (EHNA), 2-Cl-Ado, MECA, NECA	Dubey et al., 2001
	↑ proliferation	ERK1/2 (?)	Human retinal endothelial cells	NECA	Grant et al., 2001
	↑ DNA synthesis, proliferation	cAMP ?	Porcine and rat arterial endothelial cells	2-Cl-ADO, NECA, MECA	Dubey et al., 2002
	↑ DNA synthesis, proliferation	n.d.	Rat pituitary foliculostellate cells	NECA, CGS21680	Rees et al., 2002
A_3	↑ DNA synthesis	PKC, MEK, ERK1/2	Human fetal astrocytes	NECA, 2-Cl-ADO, IB-MECA	Neary et al. 1998
	↓ DNA synthesis, proliferation, cell cycle arrest, ↓ telomeric signal	n.d.	Rat lymphoma cells	Adenosine, IB-MECA	Fishman et al., 2000
	↓ DNA synthesis, proliferation, cell cycle progression	n.d.	CHO, HEK293 cells	IB-MECA, Cl-IB-MECA	Brambilla et al., 2000

synthesis in the S phase (Jones and Kazlauskas, 2001). The fact that both G_i and G_s-coupled adenosine receptors can activate the ERK1/2 and the PI3K-PKB/Akt pathway suggests the involvement of these signaling paths in adenosine's proliferative effects. Furthermore, there is the possibility that adenosine, being present in all body fluids, might act in concert with other growth factors or hormones to promote cell cycle progression.

Cooperation of adenosine receptors expressed on the same cell

When comparing adenosine receptor signaling in CHO cells with reality *in vivo*, one major difference becomes obvious, which lies in the nature of the system: We investigated signaling via a single receptor subtype, while in cells endogenously expressing adenosine receptors, several subtypes are usually co-expressed.

Table 6d: Adenosine receptor-mediated effects on mitogenesis and possible receptor subtypes and signaling pathways involved.

Adenosine receptor subtype	Effect on mitogenesis or differentiation	Proposed signaling pathway	Cellular system	Agonist used	Reference
NOT SPECIFIED	↓ proliferation	n.d.	Guinea pig thymocytes	Adenosine (EHNA), PIA	Sandberg, 1983
	↓ proliferation, ↑ differentiation	n.d.	Rat pheochromocytoma (PC12) cells	Adenosine, 2-Cl-Ado	Huffaker et al, 1984
	growth	n.d.	Explanted mouse hindlimb buds	Adenosine, R-PIA	Knudsen and Elmer, 1987
	↑ proliferation	n.d.	Bovine aortic or coronary venular endothelial cells	Adenosine	Meininger et al., 1988
	↑ differentiation	cAMP	Human peripheral blood monocytes	Adenosine	Najar et al., 1990
	↓ colony formation, growth ↑ colony formation, growth	n.d.	Human epidermoid carcinoma cells	low adenosine (A_1 ?) high adenosine (A_2 ?)	Tey et al., 1992
	↓ DNA synthesis	n.d.	Chick astrocytes, human brain capillary endothelial cells, mouse fibroblasts (Swiss 3T3), human astrocytoma cells	Adenosine	Rathbone et al., 1992
	↑ DNA synthesis, proliferation	n.d.	Human umbilical vein endothelial cells	Adenosine	Ethier et al., 1993
	↓ proliferation, cytotoxicity	n.d.	Human metastatic cell lines	NECA	D'Ancona et al., 1994
	↓ proliferation	n.d.	Mouse Sertoli-like TM4 cells	Adenosine, CHA	Shaban et al., 1995
	↓ EGF-dependent and independent proliferation	n.d.	Human keratinocytes	Adenosine	Cook et al., 1995
	↑ DNA synthesis	n.d., A_1/A_2 costimlation	Rat microglia cells	NECA, CPA, CGS21680	Gebicke-Haerter et al., 1996
	↓ proliferation	n.d.	Rat pituitary cells	Adenosine	Lewis et al., 1997
	Stellation of astrocytes	Tyrosine dephosphorylation but not cAMP nor Ca^{2+} nor PTX-sensitive G proteins, nor ERK1/2	Rat cortical astrocytes	CCPA, CGS21680, DPMA, APNEA	Abe and Saito, 1998
	↑ differentiation	n.d.	Mouse T-cells	NECA	Hamad, 1999
	↓ DNA synthesis	cAMP-dependent and independent	Trout testicular cells	Adenosine, CGS21680, NECA, R-PIA, CPA, IB-MECA	Loir, 2001

The central question is, then, how the receptors responding to adenosine, the main endogenous ligand, cooperate to achieve phenotypic changes or a physiological response. Even though all adenosine receptors – independently of G protein coupling to G_i or G_s – can mediate activation of ERK1/2 and of other MAPKs, antagonistic effects may appear e.g. on the level of cAMP. Regarding previously described interactions between adenosine receptors and dopamine receptors (Zoli et al., 1993; Dasgupta et al., 1996), even opposite effects at the receptor level are conceivable. As a result, different cell types have a possibility to fine-tune their responsiveness to adenosine by variation of receptor subtypes and expression levels. It is known, for example, that the relative abundance of adenosine A_1 receptors over A_2 receptors (or vice-versa) has considerable effects on cAMP concentrations upon increasing adenosine levels. In cells that express $A_1>>A_2$ receptors, the inhibitory A_1-mediated effects on AC will be predominant at low levels of adenosine, turning into an increasing A_2-mediated effect with increasing levels of adenosine. In contrast, in cells expressing $A_2>>A_1$ receptors, low concentrations of adenosine will lead to an increase in cAMP, whereas higher concentrations reduce cAMP levels, thus compensating for lower receptor expression level.

Another aspect of cooperation or synergy between signaling pathways is connected to the family of P2 receptors, which are activated by nucleotides. P2 receptors, especially the G protein-coupled P2Y receptor family, were shown to mediate MAPK activation (Dickenson et al., 1998; Soltoff et al., 1998; Gao et al., 1999). Some ligands at P2 receptors, such as ATP are quickly degraded to adenosine, which will consequently act on adenosine receptors with potential effects on the MAPK cascade. This cooperation between the P2 and adenosine receptors and its effect on MAPK activation has been addressed in CHO A_1 cells (Dickenson et al., 1998) and in HEK-293 cells expressing adenosine A_{2B} receptors endogenously (Gao et al., 1999), showing a clearly synergistic interaction at least between A_1 and $P2Y_2$ receptors and thus these studies expand what was known before for other signaling pathways (Gerwins and Fredholm, 1992). In addition it is important to mention that the transient stimulation by ATP in co-operation with the slightly more long-lived adenosine signal may induce MAPK signaling that is persistent enough to induce a physiological response.

In conclusion, this thesis clarifies many aspects of adenosine receptor signaling emphasizing the activation of MAPKs, but it also raises further interesting questions and prepares the ground for more explicatory experiments.

summary

1. The cellular system of Chinese hamster ovary cells stably expressing the human adenosine A_1, A_{2A}, A_{2B}, or A_3 receptor proved to be a useful model for functional and comparative characterization of adenosine receptors.
2. Comparing the ability of adenosine and inosine to modify cAMP signaling via any one of the receptors, we conclude that adenosine is indeed the endogenous ligand also at the human adenosine receptors and that inosine is ineffective at A_2 receptors. Inosine may, however, activate adenosine A_1 and A_3 receptors with low potency and efficacy under certain circumstances.
3. Adenosine A_1, A_{2A}, and A_3 receptors can – where they are abundantly expressed – be activated by physiological adenosine concentrations, whereas adenosine A_{2B} receptors require pathophysiological adenosine levels to modify cAMP production.
4. Our studies reconfirm that methylxanthines are potent antagonists at the human adenosine A_1, A_{2A}, and A_{2B} receptors, but are relatively ineffective at the human A_3 receptors.
5. All human adenosine receptors expressed in CHO cells mediate ERK1/2 phosphorylation upon NECA or adenosine stimulation in a time and dose-dependent manner.
6. When effects on cAMP production and ERK1/2 phosphorylation are compared, agonists show similar potency at adenosine A_1, A_{2A}, and A_3 receptors, but they are much more potent in activating ERK1/2 than in elevating cAMP via adenosine A_{2B} receptors. Thus, all human adenosine receptors mediate ERK1/2 phosphorylation at physiological concentrations of adenosine, at least where they are abundantly expressed.
7. Several enzyme inhibitors acting at the ATP-site of proteins, such as the adenylyl cyclase inhibitor SQ22356, the PKC inhibitor chelerythrine and the tyrosine kinase inhibitor genistein, are potent antagonists at adenosine receptors. Thus, such substances should be used carefully and the possibility of unspecific effects kept in mind.
8. Adenosine A_3 receptors mediate ERK1/2 phosphorylation via $\beta\gamma$ release from $G_{i/o}$, PI3K, Ras and MEK. This pathway is independent of cPKC, nPKC, aPKC, $[Ca^{2+}]_i$ and Src-like tyrosine kinases. Furthermore, PKB/ Akt is activated in a time- and dose-dependent manner.
9. Adenosine A_{2B} receptors mediate ERK1/2 phosphorylation in a cAMP-dependent but PKA-independent manner. Furthermore, this effect is mediated via PI3K, EGFR transactivation and MEK, but not by the small GTPase Rap1. Downstream of PI3K the PKB/ Akt is activated in a time- and dose-dependent manner. p38 phosphorylation, however, is dependent on both cAMP and PKA.
10. In CHO A_{2B} cells phosphorylation of ERK1/2, p38, CREB and PKB/ Akt is half-maximally increased by NECA at nanomolar concentrations.
11. cAMP-mediated pathways are very diverse and cell- and stimulus-specific.
12. All adenosine receptors can activate ERK1/2, but the way this is achieved is quite diverse – receptor and cell specific.

deutsche zusammenfassung

Das Nukleosid Adenosin ist in allen Zellen und im extrazellulären Raum aller lebenden Organismen vorhanden. Die Produktion von Adenosin ist eng mit dem Energiehaushalt verknüpft, d.h. die extrazelluläre Adenosinkonzentration ist umso höher, je größer der Energieverbrauch ist. Adenosin wird von Adenosindeaminase und Adenosinkinase metabolisiert. Deaminierung führt zur Produktion von Inosin, welches in ähnlichen Konzentrationen wie Adenosin auftritt. Adenosin moduliert eine Reihe unterschiedlicher Körperfunktionen, und es ist bekannt, dass es z.B. das Herz-Kreislauf- und das Nervensystem beeinflusst, als Schmerzmodulator wirksam ist, verschiedene Effekte auf Mastzellen und die Funktion des Immunsystems zeigt, usw. Diese physiologischen Effekte werden von vier pharmakologisch und biochemisch verschiedenartigen Adenosinrezeptoren, die zur Familie der G Protein gekoppelten Rezeptoren gehören, vermittelt. Die Adenosin A_1 und A_3 Rezeptoren aktivieren heterotrimere G Proteine der G_i Familie, wogegen die Adenosin A_{2A} und A_{2B} Rezeptoren G_s Proteine aktivieren. Mit dem Ziel die Pharmakologie und Signaltransduktion von menschlichen, rekombinanten Adenosinrezeptoren zu charakterisieren, wurden diese in Chinesischen Hamsterzellen (CHO Zellen) stabil exprimiert. Dies ist insofern von Vorteil, um einzelne Adenosinrezeptor-subtypen zu beschreiben, da keine ausreichend spezifischen pharmakologischen Werkzeuge vorhanden sind, und da viele Zelltypen mehr als einen Rezeptorsubtypen exprimieren. Außerdem erlaubt dieses Zellsystem uns, die verschiedenen Adenosinrezeptoren vor einem identischen zellulären Hintergrund zu vergleichen. Die humanen Adenosinrezeptoren werden tatsächlich von Adenosin aktiviert, wobei auch der Metabolit Inosin die Adenosin A_1 und A_3 Rezeptoren aktiviert, jedoch mit vergleichsweise niedriger Potenz und Effizienz. Folglich ist Adenosin der hauptsächliche Ligand an Adenosinrezeptoren, und Adenosin A_1 und A_{2A} Rezeptoren scheinen bei normalen Adenosinkonzentrationen (30-300 nM) konstitutiv aktiviert zu sein. Adenosin A_{2B} und A_3 Rezeptoren werden dagegen nur bei erhöhten Adenosinkonzentrationen stimuliert. Inosin, in hoher Konzentration, kann Adenosin A_3 Rezeptoren beeinflussen, zumindest dort wo dieser Rezeptor reichlich vorhanden ist.

Adenosin beeinflusst Zellwachstum sowohl positiv als auch negativ. Eine wichtige Rolle bei der Regulierung des Zellwachstums spielt die Familie der Mitogen-aktivierten Proteinkinasen (MAPK). In dieser Studie zeigen wir, dass alle Adenosinrezeptoren Signalwege aktivieren, die zur Phosphorylierung der MAPK Extrazelluläres Signal-regulierte Kinase 1/2 (ERK1/2) führen. ERK1/2 Phosphorylierung steigt nach Zugabe des unspezifischen Adenosinrezeptoragonisten 5´-N-ethylcarboxiamidoadenosin oder Adenosin dosis- und zeitabhängig an. Adenosinrezeptoren vermittelten die ERK1/2 Phosphorylierung mit unter-schiedlicher Effizienz: $A_3 > A_1 > A_{2A} > A_{2B}$. Die EC_{50} Werte der A_1, A_{2A} und A_3 Rezeptoren stimmten mit schon früher beschriebenen Effekten auf Adenylyl-zyklase überein, wogegen die NECA-induzierte ERK1/2 Phosphorylierung in CHO A_{2B} Zellen schon nach Zugabe von 20 nM halb-maximal war, verglichen mit halb-maximalem Anstieg der cAMP Produktion nach Zugabe von 1,4 µM NECA.

Aufgrund der hohen Effizienz und der hohen Potenz von Agonisten in CHO A_3 und CHO A_{2B} Zellen, ERK1/2 Aktivierung zu stimulieren, analysierten wir die zugrundeliegenden intrazellulären Signalwege mit Hilfe von sowohl pharmakologischen als auch molekular-biologischen Werkzeugen. Die Anwendung von Kinase- und Adenylylzyklase-inhibitoren bringt allerdings einige methodologische Probleme mit sich. Aufgrund struktureller Ähnlichkeit vermögen diese Inhibitoren, die die ATP Bindungsstelle der Enzyme blockieren, auch die Ligandenbindungsstelle von Adenosinrezeptoren zu binden und zu blockieren. Dies wurde experimentell mit Hilfe von Radioligandbindungsversuchen belegt.

Adenosin A_3 Rezeptoren aktivieren ERK1/2 indem $\beta\gamma$ Untereinheiten von pertussis-toxin-empfindlichen G_i Proteinen freigegeben, und Phosphatidylinositol-3′-Kinase (PI3K), das kleine GTP-bindende Protein Ras und die MAPK Kinase MEK aktiviert werden. G_s gekoppelte Adenosin A_{2B} Rezeptoren, andererseits, aktivieren ERK1/2 mit Hilfe von cAMP, PI3K, EGFR Transaktivierung und MEK, wogegen dieser Signalweg von cAMP-abhängiger protein kinase (PKA) und Rap1 unabhängig ist. Auf der anderen Seite werden die Stress-aktivierte Protein Kinase p38 und der Transkriptionsfaktor CREB via cAMP und PKA aktiviert.

Zusammenfassend, kann man sagen, dass diese Arbeit einige Unklarheiten im Gebiet der adenosinerezeptorabhängigen Signaltransduktion beseitigt und gleichzeitig aber auch Raum für interessante und neue Fragestellungen geschaffen hat.

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paper V