Alpha-2 Adrenergic Receptors
and Signal Transduction

Effector Output in Relation to G-Protein Coupling
and Signalling Cross-Talk

BY

JOHNNY NÄSMAN
ABSTRACT


The alpha-2 adrenergic receptor (α2-AR) subfamily includes three different subtypes, α2A-AR, α2B-AR, and α2C-AR, all believed to exert their function through heterotrimeric G\textsubscript{i/o} proteins. The present study was undertaken in order to investigate subtype differences in terms of cellular response and to explore other potential signalling pathways of α2-ARs.

Evidence is provided for a strong G\textsubscript{s}-protein coupling capability of the α2B-AR, leading to stimulation of adenylyl cyclase (AC). The difference between the α2A- and α2B-AR subtypes, in this respect, was shown to be due to differences in the second intracellular loops of the receptor proteins. Substitution of the second loop in α2A-AR with the corresponding domain of α2B-AR enrolled the chimeric α2A/α2B receptor with functional α2B-AR properties. Dual G\textsubscript{i} and G\textsubscript{s} coupling can have different consequences for AC output. Using coexpression of receptors and G-proteins, it was shown that the ultimate cellular response of α2B-AR activation is largely dependent on the ratio of G\textsubscript{i} to G\textsubscript{s}-protein amounts in the cell. Also G\textsubscript{i}- and G\textsubscript{o}-proteins appear to have different regulatory influences on AC. Heterologous expression of AC2 together with G\textsubscript{i} or G\textsubscript{o} and the α2A-AR resulted in receptor-mediated inhibition of protein kinase C-stimulated AC2 activity through G\textsubscript{o}, whereas activation of G\textsubscript{i} potentiated the activity.

α2-ARs mobilize Ca\textsuperscript{2+} in response to agonists in some cell types. This response was shown to depend on tonic purinergic receptor activity in transfected CHO cells. Elimination of the tonic receptor activity almost completely inhibited the Ca\textsuperscript{2+} response of α2-ARs.

In conclusion, α2-ARs can couple to multiple G-proteins, including G\textsubscript{i}, G\textsubscript{o} and G\textsubscript{s}. The cellular response to α2-AR activation depends on which receptor subtype is expressed, which cellular signalling constituents are engaged (G-proteins and effectors), and the signalling status of the effectors (dormant or primed).

Key words: Adrenergic, receptor, G-protein, adenylyl cyclase, cAMP, calcium, cross-talk.
The thesis is based on the following papers, which are referred to in the text by their Roman numerals:


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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>AR</td>
<td>adrenergic receptor (as in $\alpha$-AR and $\beta$-AR)</td>
</tr>
<tr>
<td>BEVS</td>
<td>Baculovirus Expression Vector System</td>
</tr>
<tr>
<td>$[Ca^{2+}]_i$</td>
<td>intracellular concentration of free $Ca^{2+}$</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMK</td>
<td>calmodulin kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary (a cell line)</td>
</tr>
<tr>
<td>Ctx</td>
<td>cholera toxin</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>$G_{x}$-protein</td>
<td>heterotrimeric GTP-binding protein with $x$ identity of $\alpha$ subunit</td>
</tr>
<tr>
<td>$G_{\alpha_x}$</td>
<td>$\alpha$ subunit of heterotrimeric G-protein with $x$ identity</td>
</tr>
<tr>
<td>$G_{\beta\gamma}$</td>
<td>$\beta\gamma$ subunit complex of heterotrimeric G-protein</td>
</tr>
<tr>
<td>i2/i3-loop</td>
<td>second/third intracellular loop of receptors</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>phosphtidylinositol</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphtidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PK</td>
<td>protein kinase (as in PKA and PKC)</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipase (as in PLC, PLA$_2$, PLD)</td>
</tr>
<tr>
<td>Ptx</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>Sf9</td>
<td>Spodoptera frugiperda (a cell line)</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain of receptors</td>
</tr>
<tr>
<td>TPA</td>
<td>12-$O$-tetradecanoyl phorbol-13-acetate</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 General background

All cells, whether single cell organisms or entities within multicellular organisms, need to sense the exterior to be able to adapt to changes. In the course of evolution, a multitude of such sensing molecules has evolved. A glimpse of this multitude comes from the realization that cells of our own bodies are regulated by a diversity of signals ranging from physical phenomena, such as light, through elementary ions, like calcium, to numerous hormones and neurotransmitters. The action of hormones and neurotransmitters is of prime importance and interest, which is reflected by the fact that the majority of drugs used in medical care are targeted toward the sites of action of these molecules. Some hormones are lipophilic and, thus, readily traverse the cell membrane to exert their effects in the cell. Other hormones, and the neurotransmitters, bind to and activate plasma membrane receptors to convey the signal to the interior of the cell. In this way, a cell will be predisposed to the action of a particular hormone only if the appropriate receptor is expressed, thereby ascertaining a level of selectivity in hormone signalling. A majority of the cell surface receptors, including the $\alpha_2$-adrenergic receptors ($\alpha_2$-ARs), belong to the superfamily of G-protein coupled receptors (GPCRs).

Intracellular signalling by GPCRs is an area that has generated a large amount of scientific knowledge during the last decades. A major breakthrough in elucidation of hormone action via receptors came from work by Earl Sutherland in the 1950s and 1960s. Studying the effect of adrenaline and glucagon on glycogen metabolism in the liver, he discovered that the hormone action on glycogen phosphorylase can be reconstituted in a cell-free system, as long as the particulate fraction of the cell material is included. This led to the discovery of adenosine cyclic 3',5'-monophosphate (cAMP), a messenger molecule between hormone receptor and target enzyme, and the identification of the enzyme responsible for its synthesis, adenylyl cyclase (AC) (reviewed in Sutherland, 1972). Since then, cAMP has been implicated, not only in metabolic regulation, but in virtually all physiological processes studied. Most of the
actions of cAMP are mediated by protein kinase A (PKA). Another ubiquitous enzyme, involved in signal interpretation, is protein kinase C (PKC). A role for PKC in intracellular signalling was not recognized until it was demonstrated that the enzyme was activated by diacylglycerol (DAG) (reviewed in Nishizuka, 1984), a product of phosphatidylinositol (PI) breakdown resulting from receptor activation (Berridge, 1987). Activation of PKC is often implicated in physiological processes such as cellular proliferation and differentiation (see e.g. Watters & Parsons, 1999). The PI breakdown also has consequences for the homeostasis of calcium ions (Ca\(^{2+}\)) in cells. A physiological role for Ca\(^{2+}\) has been known for a long time (Ringer, 1883), but many aspects of Ca\(^{2+}\) signalling by receptors, and cellular consequences of Ca\(^{2+}\) transients, are still not clarified.

cAMP, DAG, and Ca\(^{2+}\) are usually classified as second messenger molecules. The regulation of the cellular concentration of second messengers by GPCRs is fundamental to a precise regulation of the cellular machinery. One way to achieve an understanding of this precision is to elucidate the function of individual receptors in relation to the different signalling components with which they interact. The goal of the present study was to accomplish a step in this direction.

1.2 G-protein coupled receptors and signal transduction
Over one percent of the human genome encodes for more than 1000 putative GPCRs (Flower, 1999). These have probably evolved as a consequence of the variety of signals a highly developed organism needs to respond to. GPCRs are intimately involved in most physiological processes that are under hormonal regulation. In addition, synaptic transmission, vision, chemotaxis, as well as perception of taste and smell, are also governed by GPCRs. It is therefore not surprising that the same signalling theme has a long evolutionary history; the yeast pheromone response pathway regulating mating behaviour also being relayed by GPCRs and G-proteins (Blumer & Thorner, 1991).

GPCRs are, as the name implies, coupled to guanosine-5’-triphosphate (GTP)-binding proteins (G-proteins) of heterotrimeric \(\alpha, \beta, \gamma\) composition. The G-proteins are
known as 'transducers’, transducing the signals to effector proteins. The signal transition from receptor to effector is thus dependent on protein–protein interactions and is delimited to the plasma membrane. The signal is further conveyed from the effector through diffusible second messengers, enabling a spreading of the intracellular signal.

As indicated earlier, GPCR signalling is often a target for therapeutic intervention. Perturbation of normal signalling can sometimes have severe consequences. This is exemplified by the numerous activating mutations of GPCR signalling components found in different kinds of tumours (Marinissen & Gutkind, 2001). Other examples are the bacterial exotoxins of Bordetella pertussis (pertussis toxin, Ptx) and Vibrio cholerae (cholera toxin, Ctx), both of which perturb G-protein function (Milligan, 1988).

1.2.1 Receptors

The term 'receptive substance’, or receptor as we now call it, was coined by J. N. Langley in the early 20th century when studying the interaction of nicotine and curare on neuromuscular transmission (Langley, 1905). A long time passed before the ‘receptive substances’ took the form of defined structural proteins. The first GPCR to be purified, sequenced (Ovchinnikov, 1982), and eventually cloned (Nathans & Hogness, 1983) was bovine rhodopsin. Based on the predicted structural topography, with seven hydrophobic amino acid stretches presumed to adopt membrane-spanning \( \alpha \) helices, the rhodopsin protein showed similarities to bacteriorhodopsin, a bacterial proton pump for which the structure had been determined (Henderson & Unwin, 1975). However, it was not clear from the rhodopsin sequence that other receptors would have a similar structural arrangement. The purification of the \( \beta \)-ARs was accomplished in the early 1980s (Shorr et al., 1981, Shorr et al., 1982) and reconstitution experiments with receptor, G-protein, and AC confirmed the hypothesis of a three-component signalling system (May et al., 1985, Feder et al., 1986). In 1986, two independent groups reported the cloning of \( \beta \)-ARs, the turkey \( \beta_1 \)-AR (Yarden et al., 1986) and the hamster \( \beta_2 \)-AR (Dixon et al., 1986). The \( \beta \)-ARs show an overall
structural similarity to rhodopsin, with seven putative transmembrane helices (TMs) connected by hydrophilic intra- and extracellular loops, although the primary amino acid sequence homology to rhodopsin is weak.

After the successful cloning of the β-ARs, it was soon noted that GPCRs constituted a large family of similarly structured proteins. The multitude of cloned GPCRs reported during the following years set the starting point for heterologous expression and thorough investigation of receptor function. All GPCRs are believed to function through a common molecular mechanism. The binding of an agonist causes conformational changes in the receptor protein. These changes then lead to activation of G-proteins by promoting GTP for GDP exchange of the α subunit. The agonist action on the receptor protein is a poorly understood process, although direct evidence has been obtained for conformational changes (Gether et al., 1995). How the activated receptor triggers G-protein activation is also rather poorly understood (Wess, 1998).

On the other hand, molecular cloning of GPCRs provided the means for advanced structure–function studies and the mapping of receptor interaction sites. The first study on chimeric α₂/β₂-receptors, i.e. receptor hybrids with domains from both parental receptors, was reported in 1988 (Kobilka et al., 1988). It was concluded from the experiments that a particular section of the β-AR, including transmembrane helices (TM) five and six and the third intracellular loop (i3-loop) connecting the helices, is involved in the G-protein interaction. The chimeric receptor approach was soon realized to be a very efficient mean to map receptor–G-protein interactions, and a wealth of information has been gathered on this subject (see e.g. Wess, 1997).

1.2.2 Transducers

G-proteins belong to a superfamily of GTPases. A protein that transduces the signal from receptor to effector was first anticipated by Dr. Rodbell and coworkers in 1971 in light of the guanine nucleotide requirement for glucagon action on AC (Rodbell et al., 1971). The AC stimulatory G-protein, G_s, was later purified by Dr. Gilman’s group and found to be heterotrimeric, composed of an α, a β, and a γ subunit (Northup et al.,
1980). Subsequently, the AC inhibitory G-protein, G_i, was identified (Katada & Ui, 1982, Codina et al., 1983, Bokoch et al., 1984). A few years earlier, it had been shown that catecholamine action on erythrocyte membranes stimulates GTPase activity (Cassel & Selinger, 1976). Analysis of the purified components revealed that the α subunits possess the GTPase activity. In the ground state a GDP molecule is bound and the α subunit is associated with the βγ complex. Receptor activation of the heterotrimer lowers the affinity for GDP, allowing GTP to bind. Based on in vitro studies, the effect can be explained by an increase in the affinity of G-proteins for Mg^{2+}, which, in turn, promotes the nucleotide exchange (Birnbaumer & Birnbaumer, 1995). The α-GTP and βγ complex then dissociates from each other to associate with, and affect, effector proteins. The dissociation theory is widely used to explain G-protein action, although not universally accepted (see e.g. Rebois et al., 1997, Chidiac, 1998). The intrinsic GTPase activity of the α subunit then returns the G-protein to the ground state, accompanied by subunit association.

As for all other components in signal transduction, molecular cloning has revealed a diversity of G-proteins (reviewed in Gilman, 1987, Hepler & Gilman, 1992, Hildebrandt, 1997). Four major subfamilies can be outlined based on α subunit structure: G_s, G_i, G_q, and G_{12}.

The G_s subfamily contains the short and long isoforms of α_s, two splice variants of a single gene, and a closely related α_s isoform, α_{olf}. G_{olf} is primarily found in olfactory neurons and mediates the effects of odorants to a specific isoform of AC (Menco et al., 1992). G_s-proteins are ADP-ribosylated by the A (active) protomer of Ctx, rendering the α_s subunit constitutively active by stabilizing the GTP-bound form of the α subunit (Cassel & Selinger, 1977, Kahn & Gilman, 1984).

The G_i subfamily is the largest, containing three α_i subunits, two α_s subunits, α_{gust}, α_z, and the transducins α_{ir} and α_{tc}. All G_i subfamily proteins, except α_z, are substrates for Ptx-catalyzed ADP ribosylation, which uncouples the G_i subfamily proteins from receptors (Ui, 1984, Milligan, 1988). It was by the use of Ptx that G_i was
identified as the inhibitory G-protein of AC (Katada & Ui, 1982). G\textsubscript{i} subfamily proteins are also involved in intracellular Ca\textsuperscript{2+} homeostasis through inhibitory actions on voltage-gated Ca\textsuperscript{2+}-channels and stimulation of PLC\textbeta isoforms. These functions are presumably mediated by the G\beta\gamma subunits and can thus also be ascribed to other G-proteins. A clear-cut effector for G\textsubscript{o}-proteins has not been identified, despite its great abundance in the brain (Neer et al., 1984, Sternweis & Robishaw, 1984). Little is known about G\textsubscript{z}, but it may be similar in action to G\textsubscript{i} (Kozasa & Gilman, 1995).

The G\textsubscript{q} subfamily of proteins include \alpha\textsubscript{q}, \alpha\textsubscript{11}, \alpha\textsubscript{14}, \alpha\textsubscript{15}, and \alpha\textsubscript{16}, all of which activate PLC-\beta enzymes (reviewed in Rhee, 2001). Expression of G\textsubscript{16}, and the mouse orthologue G\textsubscript{15}, is restricted to cells of hematopoietic origin, whereas the expression patterns of G\textsubscript{q} and G\textsubscript{11} are wide.

The function of G\textsubscript{12} and G\textsubscript{13} is largely unknown, but they appear to possess high transforming capacity when constitutively activated (for review, see Gudermann et al., 2000).

The G\beta\gamma subunit family is made up of five \beta subunit genes and eleven \gamma subunit genes (Hildebrandt, 1997). The different possible combinations of these, and their potential selectivity for specific G\alpha subunits, may contribute to specificity in receptor signalling. However, there does not seem to be much specificity in the association of either G\beta\gamma dimers or G\alpha\beta\gamma trimers, except in a few cases (Fawzi et al., 1991, Schmidt et al., 1992, Yan et al., 1996). Initially, G\beta\gamma subunits were considered inhibitory modules of G\alpha subunits by stabilizing the inactive GDP-bound form of G\alpha (Cerione et al., 1986b). Receptor activation of G-proteins requires the heterotrimeric composition (Fung, 1983), indicating a role in receptor recognition as well. In addition, G\beta\gamma subunits are hydrophobic, partially due to a \gamma-associated prenyl group, having an important role in anchoring the G\alpha subunits to the membrane (Sternweis, 1986). The first demonstrated direct effector action of G\beta\gamma was the activation of K\textsuperscript{-}-channels in atrial myocytes (Logothetis et al., 1987). Subsequently, G\beta\gamma subunits were shown to affect several AC isoforms, PLCs of the \beta family, PLA\textsubscript{2}, and voltage-gated Ca\textsuperscript{2+}-
channels (reviewed in Sternweis, 1994). An interesting aspect of Gβγ signalling is that the β subunits contain WD40 repeats, about 40 amino acid long stretches with Trp(W)-Asp(D) dipeptide sequences (Wang et al., 1994). These structures are believed to specify protein–protein interactions by binding to pleckstrin homology (PH) domains found in a multitude of signalling molecules, including PLCs and β-AR kinases (Shaw, 1995).

1.2.3 Effectors
Classically, GPCRs are linked to effectors by G-proteins. This is not true in all cases (Hall et al., 1999). Nevertheless, a multitude of G-protein–effector interactions have been demonstrated. The widely accepted effectors, directly affected by G-proteins, include adenylyl cyclase (AC), cGMP phosphodiesterase (cGMP PDE), phospholipase C (PLC), and Ca2+- and K+-channels. Here the focus will be on AC and PLC.

1.2.3.1 Adenylyl cyclase, cAMP, and PKA
AC is a ubiquitous enzyme synthesizing cAMP from Mg2+-ATP. Before the molecular cloning era, the mammalian membrane-bound ACs were divided into a common Ca2+-insensitive isoform and a neuronal Ca2+/CaM-activated isoform. Pfeuffer and colleagues managed to purify these rare membrane protein species by the use of forskolin affinity matrix (Pfeuffer et al., 1985a, Pfeuffer et al., 1985b). In 1989, the cDNA for the type 1 neuronal isoform (AC1) was reported (Krupinski et al., 1989), and subsequently a whole family a related isoforms were identified (reviewed in Sunahara et al., 1996). The topographical structure of the ACs shows similarity to membrane transporters such as P glycoprotein and the cystic fibrosis transmembrane conductance regulator (CFTR). The protein traverses the membrane 12 times in two cassetts, adopting a pseudo-dimeric structure. The cytoplasmic regions between the cassettes and distal to the second casset make up the catalytic center, and are also the sites of regulation by G-proteins and protein kinases (Tang & Hurley, 1998). ACs are subject to many regulatory inputs, indicating that the principal control of cellular
cAMP concentration lies at the level of its synthesis, and that fine-tuning of the enzymes is central to the control.

Molecular cloning of the bovine AC1 and the successful expression in Sf9 insect cells (Tang et al., 1991) were important pioneering work for establishing regulatory properties of ACs. Heterologous expression of different isoforms of ACs has since then greatly improved our understanding of AC regulation. The mammalian ACs can be largely grouped into three subfamilies based on regulatory properties (Table 1): the Ca^{2+}/CaM-stimulated isoforms, the Gβγ-stimulated isoforms, and the Ca^{2+}-inhibited isoforms. Several AC isoforms, homologous to mammalian ACs, are also found in invertebrates (Levin et al., 1992, Iourgenko et al., 1997, Korswagen et al., 1998).

As can be seen from table 1, there is a large potential for cross-talk between traditional Ca^{2+} mobilizing receptors and cAMP signalling already at the stage of cAMP synthesis. Overall, the number of positive and negative regulators of ACs are well in balance to tightly regulate the cAMP synthesis through a variety of receptors. An enigma, in this regard, is the Gβγ-stimulated subgroup, and in particular the AC2 isoform that has been demonstrated to have high basal activity (Pieroni et al., 1995), but no inhibitory input from receptors.
Table 1. Regulators of mammalian AC isoforms.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Isoform</th>
<th>Physiological regulators</th>
<th>Stimulators</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ca(^{2+})/CaM-stimulated</strong></td>
<td>AC1</td>
<td>G(_s), Ca(^{2+})/CaM</td>
<td>G(_i), G(_o), G(\beta\gamma), CaMKIV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC3(^b)</td>
<td>G(_s), Ca(^{2+})/CaM</td>
<td>G(_i), CaMKII</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC8</td>
<td>G(_s), Ca(^{2+})/CaM</td>
<td>G(_i)</td>
<td></td>
</tr>
<tr>
<td><strong>G(\beta\gamma)-stimulated</strong></td>
<td>AC2</td>
<td>G(_s), G(\beta\gamma), PKC</td>
<td>none?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC4</td>
<td>G(_s), G(\beta\gamma)</td>
<td>PKC(^c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC7</td>
<td>G(_s), G(\beta\gamma), PKC</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td><strong>Ca(^{2+})-inhibited</strong></td>
<td>AC5(^d)</td>
<td>G(_s), PKC</td>
<td>Ca(^{2+}), G(_i), PKA, G(\beta\gamma)(^e)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC6</td>
<td>G(_s)</td>
<td>Ca(^{2+}), G(_i), PKA, G(\beta\gamma)(^e)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC9(^f)</td>
<td>G(_s)</td>
<td>Ca(^{2+})/calcineurin, G(_i)(^?)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) AC1 may not be stimulated by G\(_s\) activation *in vivo* due to concomitant inhibition by G\(\beta\gamma\) (Wayman et al., 1994), but it is stimulated *in vitro* by G\(_\alpha_s\) (Tang et al., 1991).

\(^b\) Stimulation of this isoform by Ca\(^{2+}\)/CaM is dubious, since stimulation *in vitro* requires supraphysiological concentrations of Ca\(^{2+}\) (Choi et al., 1992).

\(^c\) activated PKC reduces G\(_s\)- but not forskolin-stimulated activity (Zimmermann & Taussig, 1996).

\(^d\) Inhibition of this isoform by Ca\(^{2+}\) is questionable.

\(^e\) G\(\beta\gamma\) effect determined by cotransfection of ACs and G\(\beta\gamma\) in COS-7 cells (Bayewitch et al., 1998).

\(^f\) AC9 is usually not included in this subfamily, but it is inhibited by Ca\(^{2+}\) via calcineurin (Antoni et al., 1998).
cAMP is chemically stable, but it is degraded in cells by PDEs to the inactive metabolite 5’-AMP. There are at least 15 gene products for cyclic nucleotide PDEs, grouped into seven subfamilies (Spina et al., 1998). Some of these are specific for cAMP, some for cGMP, and some are unselective. Since the activity of most of the PDEs is regulated mainly by substrate availability, the inhibition by different natural or synthetic agents is in the focus of PDE research. Theophylline (1,3-dimethylxanthine) is the archetypal PDE inhibitor. IBMX (3-isobutyl-1-methylxanthine) is closely related to theophylline, show little subtype selectivity (Ukena et al., 1993), and often used in cell culture experiments to address AC or cAMP functions.

PKA is a multifunctional cAMP-dependent serine-threonine kinase that mediates most of the effects of cAMP (Walsh & van Patten, 1994). The PKA family of enzymes are assembled from the products of four Regulatory and, at least, two Catalytic subunit genes. The R subunits homodimerize, and each R binds one C subunit forming a tetrameric holoenzyme. Activation is triggered by cAMP binding to the R subunits and subsequent dissociation of the C subunits. Besides the tight control of the cellular cAMP concentration, which is crucial for PKA activation, the increased awareness of compartmentalization in signalling has also led to identification of multiple A Kinase Anchoring Proteins (AKAPs), providing a degree of subcellular specificity of PKA action (Dell’Acqua & Scott, 1997).

1.2.3.2 Phospholipase C, Ca²⁺, and PKC

Phospholipase Cs are a group of enzymes catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ mobilizes Ca²⁺ from intracellular depots and DAG is an activator of PKC.

In the 1970s, Ca²⁺ was being recognized as a messenger molecule of hormone action (reviewed in Rasmussen, 1970, Exton, 1981). At the same time, the involvement of phosphatidylinositol (PI) in signalling was subjected to investigation (Michell, 1975). Sir Michael Berridge pulled these findings together and showed, with the help of colleagues, that IP₃ was the missing link between receptor activation and Ca²⁺
mobilization (Streb et al., 1983, reviewed in Berridge & Irvine, 1984). This discovery set the starting point for a whole new research branch trying to characterize PLCs, IP₃ action, and Ca²⁺ signalling. Today at least 11 different isoforms of PLCs, grouped into four subfamilies, β, γ, δ, and ε, have been identified (Rhee, 2001). The PLC-β isoforms are subject to GPCR signalling through Ptx-insensitive Gq subfamily of proteins as well as through Ptx-sensitive G-protein βγ subunits. Four different isoforms of PLC-βs, β1-4, are all activated by Ga₁₆ proteins. PLC-β2 appears to be restricted to hematopoietic cells, and is likely to be the target of Ga₁₆, which also activates PLC-β1 and -β3 isoforms (Kozasa et al., 1993). Gβγ has been shown to interact with PLC-β1, -β2, and -β3, with the highest affinity for PLC-β2 (Runnels & Scarlata, 1999), by binding to the PH domain of the PLC-βs (Wang et al., 1999).

The IP₃ product of PLC action mobilizes Ca²⁺ from intracellular stores, mainly the endoplasmatic reticulum. This, in turn, evokes a capacitative inflow of Ca²⁺ from extracellular space. Ca²⁺ acts as a universal messenger involved in crucial events of life cycle, such as fertilization, muscle contraction, secretion, proliferation, metabolism, and gene expression (Berridge, 1997). It is therefore not surprising that a multitude of molecules are involved in regulating intracellular Ca²⁺ homeostasis and Ca²⁺ actions. The reviewing of these is beyond the scope of this thesis.

Dr. Nishizuka and colleagues discovered PKC in 1977 (Inoue et al., 1977). This enzyme was initially identified as a Ca²⁺-activated, phospholipid-dependent serine-threonine kinase, with no obvious relation to signal transduction. Around 1980, it was shown that DAG greatly increases the affinity of PKC for Ca²⁺ and thereby, in a sense, activates it (Takai et al., 1979, reviewed in Nishizuka, 1984). As DAG is one of the products of PIP₂ hydrolysis, it is obvious that PKC is a target for PLC-activating receptors. PKC has long been implicated in cellular transformation due to the tumour promoting activity of phorbol esters, which bind to and activate PKC (Castagna et al., 1982).
As can be anticipated, the PKC enzymes constitute a family of related proteins that differ in their tissue distribution and regulatory properties (reviewed in Hug & Sarre, 1993). They can be grouped into three subfamilies based on activation requirements: the classical PKC isoforms are activated by DAG and phorbol esters in a Ca\(^{2+}\)-dependent way, the novel PKC isoforms are activated by DAG and phorbol esters in a Ca\(^{2+}\)-independent way, and the atypical PKC isoforms are not activated by conventional GPCR signalling.

### 1.3 \(\alpha_2\)-adrenergic receptors

#### 1.3.1 A subfamily of adrenergic receptors

Noradrenaline and adrenaline exert their effects through a large family of GPCRs encoded by nine different genes in humans, three of each of \(\beta\)-ARs, \(\alpha_1\)-ARs and \(\alpha_2\)-ARs (Pepperl & Regan, 1994). The \(\alpha\)-ARs were first divided from \(\beta\)-AR by Dr. Ahlqvist in the 1940s based on agonist potency differences of evoked physiological responses (Ahlquist, 1948). Later, the \(\alpha\)-ARs were further subdivided into \(\alpha_1\)- and \(\alpha_2\)-ARs based on micro-anatomical location as pre- or postsynaptic receptors, respectively (Langer, 1974). Isotope labelling of ligands finally enabled a pharmacological distinction between these subtypes using \(\alpha_1\)-selective \([^{3}\text{H}]\)prazosin and \(\alpha_2\)-selective \([^{3}\text{H}]\)yohimbine (Starke, 1981). The original classification by Dr. Langer did not hold for very long as \(\alpha_2\)-ARs were found both on postsynaptic sites and in nonsynaptic locations, such as platelets (Berthelsen & Pettinger, 1977).

The human platelet \(\alpha_{2A}\)-AR was cloned in 1987 (Kobilka et al., 1987). The predicted protein is highly homologous to the \(\beta\)-ARs in TM regions. The intracellular domains differ from the \(\beta\)-ARs, the \(\alpha_{2A}\)-AR having a relatively long i3-loop and a short carboxyl terminus whereas the opposite is true for \(\beta\)-ARs. The expressed receptor shows similar ligand binding properties as the pharmacologically defined \(\alpha_{2A}\)-AR (Bylund, 1985), with high affinity for yohimbine and low affinity for
prazosin. Soon after cloning of the first $\alpha_2$-AR, the $\alpha_{2C}$-AR (Regan et al., 1988) and the $\alpha_{2B}$-AR (Lomasney et al., 1990) subtypes were cloned. All three subtype homologues in rat and mouse have also been cloned and sequenced (see e.g. Pepperl & Regan, 1994). The rodent $\alpha_{2A}$- and $\alpha_{2B}$-ARs are of some interest to the present study. The mouse $\alpha_{2B}$-AR subtype (Chruscinski et al., 1992) is virtually identical to the human orthologue in the TM regions, but only 64% homologous in the i3-loop, suggesting that this region need not be highly conserved for preserved functions. The i2-loops are identical between mouse and human $\alpha_{2B}$-AR.

The rodent $\alpha_{2A}$-AR has different antagonist binding properties than the human $\alpha_{2A}$-AR. This finding initially classified the rodent receptor as a new pharmacological subtype, $\alpha_{2D}$-AR (Lanier et al., 1991). The $\alpha_{2D}$-AR is now accepted as the orthologue of human $\alpha_{2A}$-AR and will be referred to as $\alpha_{2A}$-AR throughout this study. The basis for the different affinities of human and mouse $\alpha_{2A}$-ARs for yohimbine has been shown to be due, at least partially, to a single amino acid difference in TM5 (Link et al., 1992).

In the original paper by Dr. Kobilka and colleagues on chimeric $\alpha_2/\beta_2$-receptors, the TM7 of both parent receptors was strongly implicated in agonist and antagonist binding specificity (Kobilka et al., 1988). However, TM7 has an important role in intramolecular interactions, stabilizing the three-dimensional conformation of the receptor (Suryanarayana et al., 1992), and may not be directly involved in ligand interactions of the $\alpha_2$-ARs. It has been shown that a conserved aspartic acid residue in TM3 (Asp$^{113}$ in $\alpha_{2A}$-AR) provides the counterion to the positively charged amino group of catecholamine ligands (Wang et al., 1991; for review, see also Savarese & Fraser, 1992). A serine residue in TM5 (Ser$^{204}$ in human $\alpha_{2A}$-AR) is believed to interact with the para-hydroxyl group of the catechol moiety of the natural ligands (Wang et al., 1991). Another serine residue (Ser$^{200}$ in human $\alpha_{2A}$-AR) has also been implicated in ligand binding affinity and signalling potency (Wang et al., 1991, Rudling et al., 1999).
1.3.2 Physiological functions

Central effects of $\alpha_2$-AR agonists include hypotension, bradycardia, sedation, sleep induction, analgesia, mydriasis, and modulation of growth hormone release from the pituitary (reviewed in Ruffolo et al., 1993, Aantaa et al., 1995). Clonidine has long been used as an antihypertensive drug in clinical practice. Clonidine stimulation of central $\alpha_2$-ARs reduces the sympathetic outflow to the periphery, leading to a reduction in arterial blood pressure and bradycardia. The exact site of action is a matter of debate, but it appears to include several centers in the medulla including nucleus tractus solitarius, a center that receives baroreceptor input (Ruffolo et al., 1993, Aantaa et al., 1995, Nicholas et al., 1996, Singewald & Philippu, 1996).

Sedation is a common side effect of clonidine, and the $\alpha_2$-AR agonists xylasine and medetomidine are frequently used in veterinary medicine for sedation and also for analgesia. Locus coeruleus, the predominant noradrenergic nucleus in the mammalian brain, is rich in $\alpha_2$-ARs and is probably the site of action for the sedative/hypnotic effect of $\alpha_2$-AR agonists (Correa-Sales et al., 1992).

In the periphery, noradrenaline released from sympathetic nerve endings and adrenaline released from the adrenal medulla exert the effects of sympathetic stimulation by activation of adrenergic receptors located presynaptically on nerve endings, or post- and/or extrasynaptically on target tissues. As Langer pointed out in his subdivision of adrenergic receptors, $\alpha_2$-ARs are predominantly located on presynaptic nerve terminals, and when activated inhibit neurotransmitter release (Langer, 1974). Presynaptic $\alpha_2$-ARs are found on all sympathetically innervated tissues examined (Ruffolo et al., 1993). Post- and/or extrasynaptic $\alpha_2$-ARs are found in vascular beds of both arterial and venous origin. Activation of these receptors leads to smooth muscle contraction and vasoconstriction (reviewed in Langer & Hicks, 1984). It may also lead to vasodilation through increased production of nitric oxide in the endothelia (Bockman et al., 1993). The arterial $\alpha_2$-ARs are believed to be mainly extrasynaptic, responding to circulating adrenaline, whereas the postsynaptic receptors...
would be of $\alpha_1$-AR origin (Ruffolo et al., 1993). Another smooth muscle tissue with $\alpha_2$-ARs is the uterus (Hoffman et al., 1981). An intersting $\alpha_2-\beta_2$-AR cross-talk in relation to pregnancy appears to occur in this tissue (Mhaouty et al., 1995). At mid-pregnancy the myometrial $\alpha_{2A}$-AR predominates and has the ability to potentiate $\beta$-AR-stimulated cAMP synthesis. This could be a mechanism of keeping the muscles in a relaxed state. At term, the $\alpha_{2B}$-AR is upregulated and attenuates the $\beta$-AR response to provide for a forced contraction at delivery.

In several target tissues, $\alpha_2$-AR activation is associated with inhibitory functions (Ruffolo et al., 1993). In the gastrointestinal tract, secretion and motility is decreased, and in pancreas, insulin release is inhibited by beta cell $\alpha_2$-ARs. In adipose tissue, $\alpha_2$-ARs inhibit lipolysis, and in the kidney, $\alpha_2$-ARs counteract vasopressin action.

A long known target for circulating catecholamines with undisputable extrajunctional $\alpha_2$-ARs are the platelets. Adrenaline induces aggregation of platelets and potentiates aggregation induced by other agents such as ADP and thrombin (Thomas, 1967). However, the physiological benefit of $\alpha_2$-AR-mediated platelet aggregation is not clear, neither is the intracellular signalling cascade regulating it (Ruffolo et al., 1993, Keularts et al., 2000).

Most classical functions of $\alpha_2$-ARs are elucidated using clonidine, $p$-aminoclonidine, and UK14,304 as selective $\alpha_2$-AR agonists (Berlan et al., 1992, Hieble et al., 1995). These ligands are unselective for the different subtypes, and most functions have been related to the $\alpha_{2A}$-AR subtype based on prazosin insensitivity (Hieble et al., 1995). Lately, the techniques of gene knock-out or knock-in in mice have been introduced in an effort to elucidate the in vivo function of different $\alpha_2$-AR subtypes (reviewed in Kable et al., 2000). Using knock-out mice, it has been shown that the long-lasting antihypertensive effect of agonists is mediated by the $\alpha_{2A}$ subtype. Upon administration of the drugs to animals, there is an initial hypertensive pressor response. This pressor response is absent in $\alpha_{2B}$-AR knock-out mice, suggesting that
the peripheral pressor response is mediated by $\alpha_{2B}$-AR action on the vasculature. Other subtype-related effects demonstrated in knock-out mice are the salt-induced hypertension, which is abolished in $\alpha_{2B}$-AR knock-outs (heterozygotes), and the presynaptic inhibition of noradrenaline release, which appears to lack an $\alpha_{2B}$-AR component, at least in sympathetic nerves innervating the heart.

1.3.3 Cellular signalling
A diminution of cAMP synthesis through $G_i$-proteins is the most frequently observed cellular signalling function of $\alpha_2$-AR activation (Fain & Garcia-Sainz, 1980). The white adipocytes provides a good example of functional consequences of traditional inhibition of AC activity. A hormone-sensitive lipase (HSL) in these cells is responsible for triglyceride breakdown and lipolysis. This HSL is stimulated by PKA-mediated phosphorylation, following that $\alpha_2$-AR activation decreases the activity of the AC−PKA−HSL pathway and counteracts $\beta$-AR stimulation of the same (Lafontan & Berlan, 1993).

In general, though, a noticeable direct physiological consequence of $\alpha_2$-ARs mediated via inhibition of cAMP synthesis seems unusual. Events like inhibition of insulin release (Ullrich & Wollheim, 1984), inhibition of neurotransmitter release (Lipscombe et al., 1989), platelet aggregation (Haslam et al., 1978), and mitogenic signalling through MAPK (Kribben et al., 1997) all appear independent of the cAMP pathway. Cellular activation of PLC (Dorn et al., 1997), PLA$_2$ (Sweatt et al., 1986), and PLD (Jinsi et al., 1996) by $\alpha_2$-ARs is even more distant to a cAMP decrease. Nevertheless, cAMP is easily measured and AC regulation serves as the most reliable functional indicator for $\alpha_2$-AR activation.

The inhibition of cAMP synthesis by $\alpha_2$-ARs has been studied in numerous clonal cell lines, expressing endogenous or ectopic $\alpha_2$-ARs, as well as in primary cell cultures. In some cell types, the cAMP synthesis is increased upon $\alpha_2$-AR activation or exhibit U-shaped inhibitory and stimulatory regulation depending on agonist conc-
entration. Table 2 lists a number of such "anomalous" behaviours of defined receptor subtypes expressed in different cell types.

**Table 2.** Stimulatory cAMP responses via $\alpha_2$-ARs.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell type</th>
<th>Effect on cAMP</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{2A}$</td>
<td>CHO</td>
<td>U</td>
<td>(Fraser et al., 1989, Eason et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>CHW</td>
<td>↑(Ptx)</td>
<td>(Cotecchia et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>COS-7</td>
<td>↑</td>
<td>(Federman et al., 1992, Eason &amp; Liggett, 1995)</td>
</tr>
<tr>
<td></td>
<td>PC-12</td>
<td>U</td>
<td>(Duzic &amp; Lanier, 1992)</td>
</tr>
<tr>
<td></td>
<td>JEG-3</td>
<td>U$^b$</td>
<td>(Pepperl &amp; Regan, 1993)</td>
</tr>
<tr>
<td></td>
<td>HEK 293</td>
<td>↑$^c$</td>
<td>(Chabre et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Rat-1</td>
<td>U, ↑(Ptx)</td>
<td>(Sautel &amp; Milligan, 1998)</td>
</tr>
</tbody>
</table>

| $\alpha_{2B}$ | CHO | ↑(Ptx) | (Eason et al., 1992) |
| | | ↑ | (Pohjanoksa et al., 1997, Rudling et al., 2000) |
| | PC-12 | ↑ | (Duzic & Lanier, 1992) |
| | JEG-3 | ↑$^b$ | (Pepperl & Regan, 1993) |
| | S115 | ↑(Ptx) | (Jansson et al., 1994) |
| | Sf9 | ↑ | (Jansson et al., 1995) |

| $\alpha_{2C}$ | CHO | ↑(Ptx) | (Eason et al., 1992, Rudling et al., 2000) |
| | JEG-3 | ↑(Ptx)$^b$ | (Pepperl & Regan, 1993) |


$^b$ indirect assay via CREB-mediated transcription.

$^c$ cells overexpressing Gs-proteins.
In many of the studies referred to in table 2, a comparison of signalling between receptor subtypes has been made in the same cell type. With this background information, it is evident that the $\alpha_{2C}$-AR is very restricted in stimulatory cAMP effects. Comparison between $\alpha_{2A}$-AR and $\alpha_{2B}$-AR suggests that the $\alpha_{2B}$-AR is more disposed for stimulation, although comparable results for $\alpha_{2A}$-AR are obtained in some cell types.

Whereas a stimulation of cAMP synthesis is usually not seen with endogenous $\alpha_{2}$-ARs, an increase in $[\text{Ca}^{2+}]_i$ in response to agonists is observed in tissue preparations of smooth muscle (Abursto et al., 1993), in primary cultures of astrocytes (Salm & McCarthy, 1990), and in clonal cell lines expressing endogenous receptors, e.g. HEL human erythroleukemia cells (Michel et al., 1989) and NG108-15 rat neuroblastoma×glioma cells (Holmberg et al., 1998). $\text{Ca}^{2+}$ signalling is also evident in several transfected cell lines (Åkerman et al., 1996, Dorn et al., 1997, Kukkonen et al., 1998).

Modulation of ion channel activity is crucial for $\alpha_{2}$-AR-mediated inhibition of neurotransmitter release. Both K$^+$- and Ca$^{2+}$-channels have been implicated in this function (Isom & Limbird, 1988). K$^+$-channel activation and Ca$^{2+}$-channel inhibition are presumed to be dependent on protein–protein interaction and, thus, independent of cAMP (Lipscombe et al., 1989, Surprenant et al., 1992).

1.4 Other receptors utilized in the study

1.4.1 Octopamine receptors

Octopus salivary glands contain enormous amounts of octopamine (Erspamer & Boretti, 1951, Saavedra, 1974). Octopamine is also found in neuronal as well as non-neuronal tissues of most other invertebrates (reviewed in Roeder, 1999). The invertebrate octopaminergic system is thought to be homologous to the vertebrate adrenergic system. This is not only due to the structural analogy of octopamine and
noradrenaline, but also for the restricted localization of octopaminergic neurons, similar to adrenergic neurons, in CNS, release of octopamine in stress situations, and the functional consequences of the release (Roeder, 1999).

The first receptor to be characterized for octopamine was found in the CNS of the cockroach *Periplaneta americana* (Nathanson & Greengard, 1973). A classification of octopamine receptors was introduced by Dr. Evans in the early 1980s. Using the locust extensor tibiae muscle as a model, three different subtypes could be recognized on functional and pharmacological criteria, class 1, 2A and 2B (Evans, 1981). Later a pharmacologically distinct receptor subtype, class 3, was characterized in neuronal tissue of the locust (Roeder, 1992). Many of the characterized octopamine receptors seem to be coupled to AC activation (class 2 and 3 receptors), but receptors coupled to Ca\(^{2+}\) mobilization (class 1 receptors) are also found (Roeder, 1999). The Sf9 cell line is one of the few clonal cell lines expressing endogenous octopamine receptors, making it useful for studies of octopamine receptor signalling (Orr et al., 1992).

### 1.4.2 Purinergic receptors

Adenine nucleotides are released from cells either from vesicles or in response to stress or hypoxia. Cellular effects of extracellular adenine nucleotides are mediated by purinergic receptors, which are expressed, in some form, by most cell types (Ralevic & Burnstock, 1998).

A physiological effect of administered adenosine was shown more than 70 years ago (Drury & Szent-Györgyi, 1929). Much later, adenosine was demonstrated to either stimulate (Sattin & Rall, 1970) or inhibit (van Calker et al., 1978) cAMP production in brain tissue, thereby setting a need for classification into adenosine A\(_1\) and A\(_2\) receptors (van Calker et al., 1979). In the 1970s, evidence were accumulating for specific ATP receptors as well, and the purinergic receptors were classified into P\(_1\) (adenosine) and P\(_2\) (ATP) receptors (Burnstock, 1980). Now, it is generally accepted that the P\(_1\) receptors encompass four subtypes of adenosine receptors linked to stimulation (A\(_{2A}\), A\(_{2B}\)) or inhibition (A\(_1\), A\(_3\)) of AC (Fredholm et al., 1994). The P\(_2\)
receptor family contains numerous ionotropic (P2X) and metabotropic (P2Y) receptors (Fredholm et al., 1994). The P2Y receptors are mostly linked to PLC activation through Gq subfamily proteins (O’Connor et al., 1991).

1.4.3 Muscarinic receptors
The sympathetic action of noradrenaline is in most tissues counteracted by the parasympathetic action of acetylcholine on muscarinic receptors. The name for this subfamily of acetylcholine receptors comes from the plant alkaloid muscarine, a potent agonist at these receptors noticed by Sir Henry Dale some 90 years ago. Muscarinic receptors, in contrast to adrenergic receptors, are also found in invertebrates (Shapiro et al., 1989). Molecular cloning of the muscarinic receptors, in humans as well as in rodents, has revealed a family of five distinct subtypes, M1-M5 (Ashkenazi & Peralta, 1994). Expression studies of these receptors have coined a consensus view of functional coupling to the G_q subfamily (M1, M3, M5) or the Gi subfamily (M2, M4) of proteins.

1.5 Why study heterologously expressed receptors?
Examination of distinct receptor subtypes in vivo is extremely difficult for several reasons. First, in the case of adrenergic receptors, the sympathetic innervation of tissues makes it difficult to distinguish responses of pre- and postsynaptic receptors (Berlan et al., 1992). Second, the available quantities of tissues are restricted, and the ethical aspects are of high weight in experimental research involving animals. Third, single cells may contain multiple receptor subtypes with similar ligand binding properties, e.g. adipocytes may contain up to five different adrenergic receptors (Lafontan & Berlan, 1993). Fourth, ligands with sufficient subtype selectivity are not available for distinction between α2-AR subtypes. Fifth, the amount of receptors in native tissues is usually very low, reflected by the fact that an overall 80,000 -
100,000-fold purification is necessary to obtain a homogenous $\alpha_2$-AR population from human platelets (Regan et al., 1982).

Another way to study receptors is by the use of immortalized cell lines. However, the establishment of cell lines with endogenous distinct receptor subtypes is not an easy task. For $\alpha_2$-ARs, this has been a secondary profit of already established cell lines in some fortunate cases. These include human HT-29 cells, expressing $\alpha_{2A}$-AR (Bylund et al., 1988), rat NG108-15 cells, expressing $\alpha_{2B}$-AR (Bylund et al., 1988), opossum OK cells and human HepG2 cells, expressing $\alpha_{2C}$-AR (Murphy & Bylund, 1988, Schaak et al., 1997). A human cell line expressing only the $\alpha_{2B}$-AR subtype has not been found.

Heterologous expression of receptors is a way of getting unlimited amounts of single receptor subtypes in a defined setting. Most receptors appear to behave naturally also in an unnatural environment, i.e. human receptors can be expressed in non-human cells and *vice versa*, and retain their capacity to bind ligands and relay signals. Heterologous expression systems also have their limitations. One such important limitation is that the signalling machinery is defined by the cells and made up of fixed transducers and effectors. Endogenous effector proteins, such as ACs, are often heterogenous in composition (Marjamaki et al., 1997, Varga et al., 1998), and lack of selective blockers for these makes it impossible to hint particular isoforms as targets of receptor action. A way to overcome this obstacle is by genetic engineering of cells to either impair endogenous expression of isoforms or overexpress exogenous isoforms.

Heterologous expression of receptors and signalling molecules has led to the discovery of a vast diversity of receptor signalling pathways. Some of these may be physiologically redundant, or even non-existing, *in vivo*. Nevertheless, given the difficulties of examining receptors in native tissues, our current understanding of receptor structure and function would not have been possible without heterologous expression.
2 AIMS OF THE STUDY

The aims of this study were:

- to characterize receptor signalling cascades in Sf9 insect cells and to optimize the Baculovirus Expression Vector System (BEVS) for functional studies of GPCRs.

- to determine what factor(s) is responsible for the opposite coupling of $\alpha_{2A}$- and $\alpha_{2B}$-ARs to cAMP production in Sf9 cells.

- to characterize $\alpha_2$-AR signalling to specific AC isoforms.

- to characterize the Ca$^{2+}$ signalling mechanism of $\alpha_2$-ARs.

- to highlight the signalling differences between $\alpha_2$-AR subtypes.
3 MATERIALS AND METHODS

This section is a brief summary of the material and methods used in the different studies and mostly a complement to these. For more details the reader is referred to individual papers.

3.1 Cell cultures

The Sf9 cell line is a clonal cell line isolated from Sf21 cell culture, which originates from *Spodoptera frugiperda* pupal ovarian tissue (Vaughn et al., 1977). The Sf9 cell line was maintained as suspension culture in glass spinner bottles with constant stirring at 25-27°C. The cells also grow as adherent cultures and were usually seeded on tissue culture dishes for the purpose of infection with recombinant baculovirus (I, II, III, V). The buffer used during experimental conditions was Na+-based. As K⁺ is the most abundant cation in the culture medium, and in the hemolymph of lepidopteran insects, a K⁺-based medium (Vachon et al., 1995) was tested in functional assays with α₂-ARs. No significant differences in the outcome compared to the Na⁺-based buffer were detected (data not shown).

Stable α₂-AR transfectants of Chinese hamster ovary (CHO) cells (Pohjanoksa et al., 1997) were obtained through Dr. Mika Scheinin (University of Turku, Turku, Finland), and grown in MEM alpha supplemented with antibiotics and 5% foetal calf serum at 37°C (IV). The clones expressed comparable levels of the different human α₂-AR subtypes (Pohjanoksa et al., 1997, Kukkonen et al., 1998).

3.2 BEVS and infection procedures

The Baculovirus Expression Vector System (BEVS) uses the *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV, family *Baculoviridae*) to deliver genes into insect cells for efficient eukaryotic expression. There are several commercially available systems for generation of recombinant baculovirus. The basic for all of them is to replace a non-essential gene of the baculovirus genome with the gene of interest. All recombinant virus constructs used in this study are replacing the polyhedrin gene,
and use the polyhedrin gene promoter to drive the expression of the recombinant genes.

Infection of cells can be done simply by adding a small amount of virus to cell cultures and allow spreading of infection by diffusion. This method is inefficient for coexpression studies involving multiple viruses. To obtain efficient coexpression, and a synchronized infection of all cells, the infection was done by sequential overlay of cells with high-titer virus stocks (III, V). The efficiency of coexpression was tested by sequential infection with wild-type virus, producing occlusion bodies visible under light microscope, and a recombinant virus expressing Green Fluorescent Protein (GFP), which can be detected with fluorescence microscopy. Examination of cells 48 h post infection indicated coexpression in virtually all cells (> 90%).

3.3 Receptor quantification
α₂-AR expression was monitored by specific binding of [³H]RX821002 ([³H]2-methoxy-idazoxan) to cell homogenates (II) or membrane fractions of homogenates (III). [³H]RX821002 is a selective α₂-AR antagonist, that exhibits low non-specific binding, and binds with high affinity also to the rodent α₂A orthologue, in contrast to [³H]rauwolscine and [³H]yohimbine (Erdbrugger et al., 1995, Deupree et al., 1996). Saturation binding studies of the human α₂-AR subtypes expressed in Sf9 cells give affinity constant (Kₐ) values slightly under 1 nM for the α₂A-AR and around 3.5 nM for the α₂B-AR (Uhlen et al., 1998). These values are similar to the values obtained for the human subtypes in CHO cells (Pohjanoksa et al., 1997), corresponding rat subtypes in COS-1 cells, and corresponding porcine subtypes in tissue preparations (Uhlen et al., 1998).

3.4 Receptor–G-protein interactions
Pertussis toxin (Ptx) was used to ablate receptor–Gₚ-βγ-protein interactions (II, V). There has been a debate regarding Ptx sensitivity of Sf9 cell G-proteins. Some investigators have failed to abolish responses with Ptx (Vasudevan et al., 1992,
Mulheron et al., 1994), while others have not encountered problems (Richardson & Hosey, 1992, Ng et al., 1993). In the paper first describing the expression of $\alpha_{2A}$-AR in Sf9 cells (Jansson et al., 1995), Ptx significantly inhibited the receptor response, but did not abolish it. We have also noticed that the inhibition of AC2 (V) is not completely abolished by Ptx treatment when using high concentrations of UK14,304 (> 3 µM) at the human $\alpha_{2A}$-AR. Such problems have not been met with the M$_4$ receptor.

The nonhydrolyzable GTP analogue GTP$_{\gamma}$S can be used to activate G-proteins independently of receptors in membranes or permeabilized cells. When applied at low concentrations, it can also be used to monitor receptor-catalyzed G-protein activation (III). An increase in GTP$_{\gamma}$S binding to G$_{i/o}$-proteins is readily observed upon receptor stimulation. Receptor-catalyzed binding to G$_s$-proteins is more difficult to obtain, and usually has a low signal-to-noise ratio (Wieland & Jakobs, 1994). A similar problem is encountered in GTPase assay, as a result of the extremely low rate of GTP hydrolysis by G$_s$-proteins (Milligan, 1988).

### 3.5 Measurement of cAMP

G$_s$-protein coupled receptors increase AC activity, which is easily measured as an increase in the cAMP content of cells. To measure G$_i$-protein coupled receptor action, a stimulant needs to be included. Forskolin, a diterpene isolated from the plant Coleus forskohlii, is known to activate AC independently of receptor activation (Seamon et al., 1981). Agonist-dependent inhibition of forskolin-stimulated cAMP production is therefore often used as a measure of G$_i$-protein activation by receptors.

To measure cAMP accumulation (I, II, III, IV, V), cells were incubated with $[^3H]$adenine to label the ATP pool, from which cAMP is formed. Cells were then stimulated, whereafter the cAMP was extracted, separated from other labelled contaminants by ion exchange chromatography and adsorption to insoluble aluminium oxide salt, and quantified by liquid scintillation counting. The method lacks spatial resolution of cAMP concentrations, but gives highly reproducible results of total cellular cAMP accumulation. The absolute concentration of cAMP cannot be obtained
by this method, since the radiolabelled cAMP (and ATP) makes up only part of the total cellular cAMP content. The cAMP level is instead related to the labelled ATP and ADP pools, eluted from the ion exchange step (Krishna et al., 1968), and gives a relative measure of the amount of conversion of ATP to cAMP.

3.6 Measurement of \( \text{Ca}^{2+} \)
Fura-2, a fluorescent probe highly selective for \( \text{Ca}^{2+} \), is delivered into cells as an acetoxymethyl ester conjugate (Fura-2 AM) (Grynkiewicz et al., 1985). The fura-2 molecule is trapped inside the cells after the removal of the AM group. Fura-2 shows a spectrum shift upon binding \( \text{Ca}^{2+} \) ions, 380 nm being the wavelength for maximal excitation in the absence and 340 nm in the presence of \( \text{Ca}^{2+} \), providing the basis for measurements. The affinity constant for the fura-2–\( \text{Ca}^{2+} \) complex at 37°C, and physiological salt concentrations, is \( \sim 220 \) nM (\( \sim 280 \) nM at 22°C), setting the sensitivity and the limitations of the probe for \( \text{Ca}^{2+} \) changes. Most cell types can be assayed for \( \text{Ca}^{2+} \) signalling in suspension using fura-2 (I, IV). However, suspension measurements do not provide information about cell heterogeneity nor spatial resolution of signalling. In addition, perfusion of cells is not technically possible. For the above purposes, the fura-2-loaded cells, on cover slips, were excited under microscopic view (IV).

3.7 Mathematical modelling
A mathematical model of receptor-regulated AC activity was developed to account for promiscuous \( G_i/G_s \)-protein coupling (III). The model includes three different steps. The first step describes the agonist activation of the receptor, in which agonist concentration and efficacy can be varied to give different concentrations of activated receptor. The second step describes the activation of two different G-proteins, \( G_i \) and \( G_s \), by the activated receptor from the first step. In the second step, the outcome, i.e. relative concentrations of activated \( G_i \) and \( G_s \), can be modified by varying the receptor’s affinity for the different G-proteins as well as by varying mutual competitiveness between the G-proteins for activation by the receptor. The third step
describes how Gi and Gs allosterically modulate AC to either decrease (Gi) or increase (Gs) the catalytic activity.

Although sufficiently sophisticated to enable the modelling work performed in paper III, the model suffers from some limitations and a refined model has been described after the publication of paper III (Kukkonen et al., 2001).

4 RESULTS

4.1 Receptor signalling in Sf9 cells
Stimulation of Sf9 cells with octopamine led to an elevation of [Ca$^{2+}$]$_i$ and cAMP production (I). The potency of octopamine was similar for both events. The [Ca$^{2+}$]$_i$ increase originated from intracellular stores as well as from the extracellular space. Activation of the expressed human adenosine A$_{2A}$ receptor, linked to Gs-proteins, resulted in cAMP elevation with no concomitant increase in Ca$^{2+}$, excluding the role of cAMP in Ca$^{2+}$ mobilization. When Ca$^{2+}$ was chelated with EGTA, there was an attenuation of the cAMP increase in response to octopamine, suggesting a role for Ca$^{2+}$ in AC activation. However, activation of the muscarinic M$_3$ receptor, linked to Gq-proteins, increased [Ca$^{2+}$]$_i$, but had no effect on basal cAMP production. Using a panel of octopamine receptor/α$_2$-AR antagonists, we found that the dual octopamine responses are mediated by different receptor populations in Sf9 cells.

It was not tested whether octopamine increases the IP$_3$ production, indicative of PLC activation. The muscarinic Gq-protein coupled receptors do increase the IP$_3$ concentration in Sf9 cells (Kukkonen et al., 1996). It is also interesting in this context to note that the heterologously expressed human α$_{2B}$-AR is coupled to IP$_3$ production and [Ca$^{2+}$]$_i$ increase in Sf9 cells (Holmberg et al., 1998), and apparently uses the same dual coupling mechanism as the octopamine receptors to increase cAMP production (Figure 1).
Figure 1. Effects of Ca\textsuperscript{2+} chelation on α\textsubscript{2B}-AR-mediated cAMP production.
Sf9 cells expressing recombinant human α\textsubscript{2B}-AR were assayed for noradrenaline (NA)-stimulated cAMP production in the presence of 1 mM Ca\textsuperscript{2+} or in the presence of 1 mM EGTA. Some cells were pretreated with ionomycin (Iono) in the presence of EGTA to deplete cells of Ca\textsuperscript{2+}. Experiments were performed in the presence of 30 µM forskolin. The concentration of NA was 100 µM. Data are means ± SEM from three different experiments performed in triplicate.

The endogenous AC(s) in Sf9 cells integrated two signals, G\textsubscript{s} and Ca\textsuperscript{2+}, to increase the total cAMP synthesis. How this is mechanistically regulated is at present unknown. Comparison to the mammalian AC1, which is directly activated by the Ca\textsuperscript{2+}/CaM complex (Tang et al., 1991), indicated that the Sf9-AC is regulated in a different manner, requiring a primary stimulant for Ca\textsuperscript{2+} effects.

In paper V we investigated receptor regulation of the mammalian AC2, heterologously expressed in Sf9 cells. The PLC-linked M\textsubscript{3} receptor was able to stimulate this AC isoform via PKC. The PKC isozyme(s) involved probably belong to
the classical subfamily based on phorbol ester (TPA) sensitivity (V) and a Ca\(^{2+}\)-dependence (data not shown).

All \(\alpha_2\)-AR subtypes can inhibit forskolin-stimulated Sf9-AC activity (Oker-Blom et al., 1993, Jansson et al., 1995, II, III, V). In the case of human \(\alpha_{2B}\)-AR, the inhibition was increased when coexpressed with mammalian G\(_i\)-proteins (III). The complex regulation of AC2 by G\(_o\)-proteins, with both inhibitory and stimulatory input depending on primary stimulus, was also obtained with the endogenous G-proteins (V).

### 4.2 Propensity of \(\alpha_2\)-ARs for G\(_s\) coupling

In paper II we investigated if the opposite coupling of the heterologously expressed \(\alpha_{2A}\)- and \(\alpha_{2B}\)-ARs to cAMP production (Jansson et al., 1995) resided in the receptor proteins as sequence differences. The mouse orthologues of these subtypes were expressed in Sf9 cells at similar densities. The \(\alpha_{2B}\)-AR action on cAMP production was U-shaped, with inhibition at low and potentiation at higher noradrenaline concentrations. The \(\alpha_{2A}\)-AR inhibited the AC activity in the equivalent concentration range. The potentiating effect of the \(\alpha_{2B}\)-AR was hypothesized to result from promiscuous coupling to G\(_s\)-proteins and, if so, ought to involve cytoplasmic domains of the receptor. An exchange of the i3-loops between the subtypes did not alter the responses of the parent receptors. The carboxyl terminal domain neither seemed to be involved. Of the remaining cytoplasmic domains, the i2-loop from \(\alpha_{2B}\)-AR conferred the stimulatory effect to \(\alpha_{2A}\)-AR. Only a few amino acid residues of the i2-loops differ between the subtypes. Site-directed mutagenesis of single amino acid residues in the i2-loop of \(\alpha_{2A}\)-AR was not enough to convincingly demonstrate an \(\alpha_{2B}\)-AR functionality, but a double mutant (S\(^{134}\)→A, L\(^{143}\)→S) showed an enhanced stimulatory capacity, suggesting that several amino acid residues are involved in the coupling specificity.

In paper III we wanted to provide evidence for true G\(_s\)-protein coupling of \(\alpha_{2B}\)-AR, and get insight into the molecular mechanisms that define the U-shaped
regulation of cAMP production. For these purposes, we coexpressed G\textsubscript{i}- or G\textsubscript{s}-proteins together with the human \(\alpha\textsubscript{2B}-\text{AR}\) in Sf9 cells. An interaction between G\textsubscript{s} and \(\alpha\textsubscript{2B}-\text{AR}\) was demonstrated by enhanced GTP\(\gamma\)S binding to G\textsubscript{s}-proteins upon receptor activation. An increase in the potency of noradrenaline to stimulate cAMP production, and an increased maximal response, were also evident with G\textsubscript{s} coexpression, indicative of an interaction in the cellular milieu as well.

The \(\alpha\textsubscript{2}-\text{ARs}\) are coupled to an inhibition of cAMP synthesis in many cell types. An increase in the G\textsubscript{i}-protein level should thus, in the case of \(\alpha\textsubscript{2B}-\text{AR}\), if not displace, then at least counteract the effect of G\textsubscript{s} on AC. G\textsubscript{i} coexpression did attenuate the forskolin stimulation via \(\alpha\textsubscript{2B}-\text{AR}\), but part of the stimulatory component remained. However, the potentiating effect with noradrenaline could be overcome with longer infection times, when the concentration of G\textsubscript{i}-proteins was prominently increased (Figure 2).

There was an obvious difference in the activation mode of \(\alpha\textsubscript{2B}-\text{AR}\) by different agonists. Noradrenaline produced U-shaped dose-response curves, while UK14,304 elicited the stimulatory phase only weakly in the same concentration range (II, III). The phenomenon, whereby different agonists can induce different responses through the same receptor, has been termed agonist-specific coupling (Robb et al., 1994). As can be seen from figure 2, this can have a major impact on AC regulation via promiscuous G\textsubscript{s}/G\textsubscript{i}-protein coupling. In this context, it is worth pointing out that the partial agonist clonidine, with apparent low intrinsic efficacy for G\textsubscript{s} coupling, induces inhibitory responses that are "fuller" than those of certain "full agonists".
Figure 2. Ligand activity at $\alpha_{2B}$-AR coexpressed with $G_{i}$-proteins.

Sf9 cells expressing recombinant human $\alpha_{2B}$-AR alone or together with $G_{i}$-proteins were assayed for agonist-mediated changes in forskolin-stimulated cAMP production at different times post infection (p.i.). The agonists were noradrenaline (NA), oxymetazoline (Oxy), UK14,304 (UK) and clonidine (Clon). The concentrations used were 100 $\mu$M for all agonists. Part of these data are presented in paper III, table 1. Data are means ± SEM (means ± SD, 38 h p.i.) from three different experiments (one experiment, 38 h p.i.) performed in triplicate.

4.3 Signalling cross-talk

The AC activity in Sf9 cells gave an example of cross-talk between different receptors at the effector level. Two distinct signal pathways, elicited by different octopamine receptor populations, activating different G-proteins, converge on the AC activity to amplify the cAMP increase (I). In this system, $G_{s}$-protein activation, by one type of receptor, has an obligatory role for the potentiating effect of Ca$^{2+}$ on the AC activity. In paper IV it was shown that activation of purinergic $G_{q}$-coupled receptors was, in a similar way, obligatory for $\alpha_{2}$-AR-mediated increase in [Ca$^{2+}$], in CHO cells. Stimulation of $\alpha_{2}$-ARs resulted in an increase in [Ca$^{2+}$], of about 200 nM. Treatment of cells with apyrase to hydrolyze extracellular purine nucleotides significantly reduced the $\alpha_{2}$-AR-mediated [Ca$^{2+}$], increase, as did the block of P$_{2Y}$ receptors with suramin. Neither apyrase nor suramin unspecifically influenced the G-protein coupling
of α₂-ARs, as judged from the AC inhibition. With continuous perfusion of cells, to remove released purine nucleotides, the Ca²⁺ response to α₂-AR stimulation was virtually absent, indicating that activation of Gq subfamily proteins is a prerequisite for PLC activation through α₂-ARS.

Among the α₂-ARs, only the α₂B-AR subtype mediates a Ca²⁺ response in Sf9 cells (Holmberg et al., 1998). Continuous perfusion of these cells did not attenuate the α₂B-AR-mediated Ca²⁺ response (Holmberg et al., 1998). In an effort to elucidate the mechanism for the Ca²⁺ response in this cell type, we coexpressed the α₂B-AR with Gᵢ- or Gₛ-proteins. The Ca²⁺ response was attenuated about 70% with Gᵢ coexpression, whereas Gₛ had no major influence on the response (Figure 3).

Figure 3. Effect of G-protein expression on α₂B-AR-mediated Ca²⁺ signalling.
Sf9 cells expressing recombinant human α₂B-AR alone or together with Gᵢ- or Gₛ-proteins were assayed for changes in [Ca²⁺]ᵢ in response to noradrenaline (1 µM). Data are means ± SD from two experiments.
In paper V another example of cross-talk between receptors was demonstrated. PKC activation, by TPA or M₃ receptor activation, elevated the cAMP content of Sf9 cells expressing AC2, but not of wild-type or AC1 expressing cells. The stimulation was almost totally inhibited by activation of the α₂A-AR or the M₄ receptor expressed in the same cells. Since AC2 has been considered refractory to inhibitory G-proteins, this led us to further investigate this apparent discrepancy. We could show that concomitant activation of Gₛ-proteins switches the response of α₂A-AR to stimulatory, presumably an effect via Gβγ, as it was sensitive to Gβγ scavenging by Gα₁₁. To identify the G-protein species mediating the inhibition, we developed a method that take advantage of the viral turn-off of host protein synthesis. This enabled us to eliminate most of the receptor coupling to endogenous G-proteins by what we call "time-restricted pertussis toxin pretreatment", i.e. Ptx-sensitive endogenous G-proteins were uncoupled by Ptx pretreatment prior to infection with viruses coding for signalling components, including G-proteins. Since Ptx was removed soon after infection, the newly synthesized G-proteins could escape ADP ribosylation. Using this approach, it was shown that Gₛ-proteins could inhibit TPA-stimulated activity and potentiate Gₛ-stimulated activity of AC2. Expression of Gᵢ, on the other hand, potentiated both of these primary stimulus events. The identity of Gβγ subunits were the same for both G-protein species, indicating that the identity of Gα subunit determines the outcome.

5 DISCUSSION

5.1 Suitability of Sf9 cells for receptor characterization
Early reports on GPCRs in Sf9 cells described the successful expression of different receptors, and purification of functional receptors from these cells (George et al., 1989, Parker et al., 1991, Reilander et al., 1991, Vasudevan et al., 1991). Expression of GPCRs in Sf9 cells ususally results in high receptor densities, well suited for studying pharmacological binding characteristics (Parker et al., 1991, Mouillac et al., 1992, Quehenberger et al., 1992, Mills et al., 1993, Oker-Blom et al., 1993, Hartman
IV & Northup, 1996). The use of Sf9 cells for functional studies became somewhat dubious by a report stating an absence of $G_i$-proteins in these cells (Quehenberger et al., 1992). Nevertheless, soon afterwards, inhibition of Sf9-AC activity via the 5-HT$_{1B}$ receptor (Ng et al., 1993) and via the $\alpha_{2C}$-AR (Oker-Blom et al., 1993) was reported. We have later shown that all three $\alpha_2$-AR subtypes can inhibit forskolin-stimulated AC activity in Sf9 cells (Jansson et al., 1995, II, III). In the case of the human $\alpha_{2B}$-AR, the inhibition was increased when coexpressed with mammalian $G_i$-proteins, suggesting Sf9-AC sensitivity toward this G-protein as well (III).

The inhibitory G-proteins in Sf9 cells have not been characterized in detail, but at least a $G_o$-like G-protein appears to be expressed (Ng et al., 1993, Mulheron et al., 1994). $G_o$-proteins are expressed predominantly in neural tissues, but are also found in other places. The $G_{o_s}$ subunit identified in Drosophila melanogaster is also expressed in oocytes and ovarian nurse cells, in addition to its neuronal localization (Schmidt et al., 1989). Having in mind that Sf9 cells originate from ovarian tissue, it is not unlikely that a Spodoptera $G_o$ is expressed in these cells. The endogenous inhibitory G-protein can obviously substitute for the mammalian $G_o$-protein for the inhibition of AC2 (V), supporting this hypothesis.

Sf9 cells have often been used to address G-protein selectivity of various receptors by expression of selected receptor–G-protein pairs (Butkerait et al., 1995, Grunewald et al., 1996, Hartman IV & Northup, 1996, Barr et al., 1997, III). This has, on some occasions, led to confirmation of presumptive G-protein coupling of receptors (Butkerait et al., 1995, Barr et al., 1997, III). One reason for utilizing Sf9 cells for establishing receptor–G-protein coupling preferences is that relatively high levels of expression of desired proteins are obtained, improving the signal-to-noise ratio. Another feature of Sf9 cells is the low level of endogenous GPCRs interfering with the ectopic receptors (Hu et al., 1994, Butkerait et al., 1995, Barr et al., 1997), as well as low levels of endogenous G-proteins functionally coupled to expressed receptors, as determined by lack of GTP-sensitive high-affinity agonist binding sites (Quehenberger et al., 1992, Butkerait et al., 1995, Grunewald et al., 1996).
Assessment of signalling pathways in whole cells could have some advantages of the above notions. For example, purinergic receptor signalling is obviously "disturbing" the cellular signalling status when working with isolated cell cultures (IV, Florio et al., 1999, Ostrom et al., 2000). We have not detected changes in cAMP production, or Ca^{2+} homeostasis, with the purinergic receptor agonists ATP and adenosine in uninfected Sf9 cells, or in cells infected with wild-type virus. This suggests that Sf9 cells could be a cell line of choice when such tonic signalling is not to interfere with the experimental outcome. Potentially interfering receptors expressed by the Sf9 cells are the octopamine receptors (Orr et al., 1992, Hu et al., 1994, I). This is especially relevant in adrenergic receptor research due to the structural similarity of ligands acting on receptors from these families. It has also been shown that octopamine activates α_{2}-ARs (Airriess et al., 1997, Rudling et al., 2000). When tested on uninfected Sf9 cells, noradrenaline had an effect on the [Ca^{2+}]_{i} and cAMP production at high concentrations (> 3 uM) (Figure 4). The effect of clonidine was prominent on the [Ca^{2+}]_{i} level, but modest on cAMP accumulation. UK14,304 did not seem to activate the endogenous octopamine receptors (data not shown), and could be the agonist of choice for studying α_{2}-ARs. Unfortunately, UK14,304 appears not to activate the α_{2}-ARs in the same manner as noradrenaline (see Figure 2).
Figure 4. Ligand activity at octopamine receptors in Sf9 cells. Uninfected Sf9 cells were assayed for α2-AR agonist-mediated changes of cAMP accumulation or [Ca^{2+}]_i. The agonists were: noradrenaline (NA), oxymetazoline (Oxy), clonidine (Clon) and p-octopamine (Oct). The concentrations used were 100 µM for all agonists. The data for cAMP accumulation are given as changes from basal level and are means ± SD, N=3. Data for [Ca^{2+}]_i are given as changes from basal level and are means ± SD, N=2.

A drawback of the BEVS is that the viral infection is lethal to cells. This renders the Sf9 cells unable to maintain ionic gradients and metabolic equilibrium status late in infection. A primary reason for this deterioration is the virally mediated turn-off of host protein synthesis. However, this can be turned to an advantage in receptor research by Ptx pretreatment prior to infection to abolish receptor coupling to endogenous G-proteins, and subsequent recovery of signal pathways by heterologous expression of desired G-proteins (V).

5.2 Promiscuous G-protein coupling

A receptor is primarily coupled to a specific subset of G-proteins and activation of these by the receptor is efficacious and occurs with high agonist potency. For example, pioneering work with purified platelet α2-AR and G-proteins, reconstituted in phospholipid vesicles, revealed that this receptor activates G_i and G_o very efficiently, whereas activation of G_i and G_s is negligible (Cerione et al., 1986a). It has later been shown that many receptors are not that strict in selecting G-protein partners, but
instead they exhibit additional coupling to G-proteins from distantly related subfamilies (Gudermann et al., 1996). It is this phenomenon that is usually referred to as promiscuous G-protein coupling.

Activation of $\alpha_2$-ARs results in a seemingly controversial stimulation of cAMP synthesis in some cell types. This phenomenon was initially shown to be mediated by PLA$_2$ activation (Jones et al., 1991), but subsequent studies also revealed a probable G$_s$-protein interaction of the $\alpha_2A$-AR in transfected CHO cells (Eason et al., 1992). In Sf9 cells, the $\alpha_2B$-AR is linked to an increase in cAMP production, whereas the $\alpha_2A$-AR subtype decrease forskolin-stimulated AC activity (Jansson et al., 1995). A productive G$_s$-protein coupling of the $\alpha_2B$-AR in these cells was demonstrated in paper III. Work from Dr. Liggett’s lab has suggested that there are subtle differences in the propensities of the different $\alpha_2$-AR subtypes for G$_s$ activation with the endogenous catecholamine ligands (Eason et al., 1994). Our initial studies pointed to an all-or-none difference between $\alpha_2B$-AR, on the one hand, and $\alpha_2A$-AR and $\alpha_2C$-AR on the other (Oker-Blom et al., 1993, Jansson et al., 1995). A true difference between the $\alpha_2A$- and $\alpha_2B$-AR subtypes, although not as dramatic, was demonstrated in paper II, in which the coupling differences cannot be ascribed to differences in receptor densities. It now seems clear that the $\alpha_2B$-AR subtype is more propensive for G$_s$ coupling than the other subtypes (Eason & Liggett, 1993, Pepperl & Regan, 1993, II, Rudling et al., 2000, III). The weak capability of the $\alpha_2A$-AR to stimulate cAMP production in the presence of surplus G$_s$-proteins also supports this notion (III).

The structure of the i2-loop appears to be a determinant for this subdivision (II). The whole selection structure for G$_s$ coupling is probably not confined to this area, since potent G$_s$ activation is also obtained with $\alpha_2A$ in different cell types (see Table 2). The first study on this issue showed that deletion of the amino terminus of the i3-loop of $\alpha_2A$-AR eliminates stimulation of AC activity, indicating a G$_s$ coupling domain in this area (Eason & Liggett, 1995). However, substitution of this domain with corresponding domain from $\beta_2$-AR, the prototype G$_s$ coupled receptor, attenuated the G$_s$ coupling when compared with the intact $\alpha_2A$-AR (Eason & Liggett, 1995). On
the other hand, substitution of the i2-loop with β2-AR sequence significantly improved the Gs coupling (Jewell-Motz et al., 1997), supporting our hypothesis about the importance of i2-loop structure for Gs coupling (II).

The dose-response curve forms for α2-AR action on AC activity varies a lot from cell type to cell type and from receptor subtype to receptor subtype, including U-shaped relations in addition to monophasic stimulatory and inhibitory curves. Even more intriguing, all these different forms of dose-response curves can be obtained in a single cell type with a single receptor subtype (III). To be able to explain these behaviours as a simple consequence of dual Gi and Gs coupling, we developed a mathematical model that monitor AC activity in relation to Gi and Gs activation (III). Using this model, it was possible to reproduce the different curves obtained in experiments, suggesting that relative affinities of the receptor for Gi and Gs activation can alone explain the different behaviours. The results from the modelling also suggested that different agonists, like noradrenaline and UK14,304, differentially activate the receptor and thereby dictate relative affinity differences of the receptor for the two G-protein species.

It is difficult to correlate the promiscuous G-protein coupling of α2-ARs to a specific physiological effect. It is clear though, that a decrease in cAMP synthesis cannot explain many effects of α2-AR activation observed in vivo (Isom & Limbird, 1988). A comment on recent progress in β-AR research on this topic may be valid here. Prior to cloning of the β-AR, purification enabled reconstitution of this receptor with G-proteins. From these experiments it was concluded that the β-AR cross-reacted with Gi (Cerione et al., 1985). A couple of years ago, it was shown that β-ARs evoke a Ca\(^{2+}\) signal in submandibular gland cells through a mechanism involving activation of Gi-proteins (Luo et al., 1999). Very recently, Dr. Kobilka’s group provided evidence for promiscuous coupling of β2-AR to Ptx-sensitive G-proteins in myocyte cultures from β1-AR knock-out mice, the result of which was recorded as a decrease in contraction rate (Devic et al., 2001). These studies implicates that promiscuous coupling may be a general mode of diversifying receptor signalling and not simply a
laboratory phenomenon due to high expression levels of receptors, or abnormal signalling environments of used cell types.

5.3 Adenylyl cyclase regulation by the Ca^{2+} signalling pathway

AC regulation by Ca^{2+}/CaM is perhaps the most well conserved regulatory theme of cAMP synthesis. Pathogenicity of *Bacillus anthracis* and *Bordetella pertussis* is partially due to secreted, soluble Ca^{2+}/CaM-stimulated AC enzymes (Danchin, 1993). In higher organisms, Ca^{2+}-regulated AC activity is particularly associated with neural functions (Dudai & Zvi, 1984, Xia & Storm, 1997, Chern, 2000). In Sf9 cells, the AC activity was not stimulated by an increase in [Ca^{2+}]_i, but the primary, Ca^{2+}-independent, cAMP elevation with octopamine, and with α_{2B}-AR activation, was highly potentiated by a concomitant increase in [Ca^{2+}]_i. The potency of forskolin for AC stimulation was similar in the absence and presence of elevated [Ca^{2+}]_i, suggesting that Ca^{2+} does not alter the affinity for the primary stimulant. A likely explanation is that Ca^{2+} increases the activity of the enzyme, integrating the Ca^{2+} signal with the direct activator. Signalling molecules with the property of integrating multiple signals are called coincidence detectors (Iyengar, 1996). Coincidence detection of ACs is thought to play important roles in transcription, synaptic plasticity and endocrine function.

AC2 is a coincidence detector for G{alpha}s and PKC as well as G{beta}{gamma} subunits (reviewed in Sunahara et al., 1996). A cross-talk between PKC and AC was suggested in 1987 in a paper showing phorbol ester-mediated phosphorylation of AC in erythrocytes (Yoshimasa et al., 1987). However, it is still unclear what physiological role PKC play in cAMP signalling. AC2 has been suggested to play a role in synaptic plasticity (Defer et al., 2000), although this has never been demonstrated. The AC2 isoform has in several instances been implicated in growth and development. In NIH-3T3 cells, ectopic expression of AC2 is sufficient to inhibit proliferation in response to serum (Smit et al., 1998). Neuronal, as well as mesodermal, differentiation of the teratocarcinoma P19 cells is accompanied by an upregulation of AC2 mRNA (Lipskaia et al., 1997, Lipskaia et al., 1998). A related isoform of the mammalian AC2
is expressed in *Caenorhabditis elegans*. Loss of function of this isoform results in early larval lethality, indicating an important role in development (Korswagen et al., 1998).

In mammals, AC2 is widely expressed in the brain with no specific area preference (see e.g. Mons & Cooper, 1995). This suggests a role in common neuronal action or development. In light of the results presented in paper V, it is tempting to speculate, as follows, about such a common action. Neurotransmitter release is a complicated process triggered by \([\text{Ca}^{2+}]_i\) increases. The amount of \(\text{Ca}^{2+}\) entering the nerve terminal is strictly regulated by voltage-gated \(\text{Ca}^{2+}\)-channels. Some of these channels are targets for PKC-mediated upregulation of activity (Zamponi et al., 1997), whereas attenuation is transmitted from GPCRs, such as \(\alpha_2\)-ARs, via activation of \(G_{\alpha}\) or \(G_\gamma\)-proteins (for review, see e.g. Dolphin, 1998). Recent studies have implicated a role also for cAMP in the neurotransmitter release process (Trudeau et al., 1996, Chavis et al., 1998, Chang et al., 2000). Thus, if AC2 and \(\text{Ca}^{2+}\)-channels were colocalized in the nerve terminals, both a coordinated stimulation of AC2 and \(\text{Ca}^{2+}\)-channels via PKC, or a coordinated inhibition of the same molecules via \(G_{\alpha}\)-proteins, could account for synergistic effects on transmitter release.

### 5.4 The \(\alpha_2\)-AR–\(\text{Ca}^{2+}\) link

There are now compelling evidence that \(\alpha_2\)-ARs can alter the \([\text{Ca}^{2+}]_i\) by other mechanisms than through ion channel modulation. Activation of \(\alpha_2\)-ARs in a variety of cell types mobilizes \(\text{Ca}^{2+}\) from intracellular stores (Michel et al., 1989, Salm & McCarthy, 1990, Åkerman et al., 1996, Dorn et al., 1997). In light of the multitude of \(G_{i\alpha}\) coupled receptors that have been shown to mobilize \(\text{Ca}^{2+}\), it is no wonder that the \(\alpha_2\)-ARs behave similarly. It was early reported that, for some receptors, the link to \(\text{Ca}^{2+}\) mobilization and PLC activation is sensitive to Ptx (see e.g. Ui et al., 1988). The finding that PLC-\(\beta\)s are stimulated by \(G\beta\gamma\) subunits gave a satisfactory explanation to this phenomenon. For the \(\alpha_2\)-ARs, a \(G\beta\gamma\)-dependent \(\text{Ca}^{2+}\) mobilization has been demonstrated in COS-7 cells (Dorn et al., 1997).
Characterization of PLC-β isoforms in vitro suggested a direct activation by Gβγ, without conditioning by Gq subfamily proteins (Camps et al., 1992, Katz et al., 1992). In CHO cells, expressing either subtype of α2-ARs, the α2-AR-mediated Ca2+ mobilization was highly dependent on tonic stimulation of Gq coupled receptors (IV). This led us to hypothesize that Gβγ-mediated PLC signalling requires concomitant αq/11/14/16 activation. The mechanistic explanation has been elaborated in a study by Dr. Wong and colleagues, showing that Gi-protein coupled opioid receptors could not activate PLC, unless coexpressed with Gα16, in COS-7 cells (Chan et al., 2000). On the same theme, they showed that the opioid receptors acquire the ability to stimulate PLC in cells cotransfected with constitutively activated Gαq or Gα14. Further support for the relevance of cross-talk or promiscuous G-protein coupling in Ca2+ signalling has appeared from two papers describing signalling in knock-out mice deficient in Gq family proteins. First, PLC activation by thrombin, a Gq and Gi coupled receptor, is abolished in platelets from Gαq-deficient mice, indicating that Gi activation alone is insufficient for PLC activation (Offermanns et al., 1997). Second, the C5a receptor, which is strongly coupled to Ca2+ mobilization through Ptx-sensitive G-proteins, looses the Ca2+-mobilizing ability in macrophages from Gα15 knock-out mice, while MAPK activation is normal (Davignon et al., 2000).

Most Gi-protein coupled receptors in hematopoietic cells, such as the C5a receptor described above, usually respond to agonists with robust [Ca2+]i increases. Hematopoietic cells express the Gα15/16 subtype of G-proteins (Amatruda et al., 1991). The G15/16-protein has been found rather unselective for receptor interactions (reviewed in Milligan et al., 1996). Thus, if there is promiscuous receptor coupling to G15/16, the Ca2+ response should largely benefit from this. In HEL cells, α2-ARs give prominent Ca2+ responses (Michel et al., 1989, Kukkonen et al., 1997, Jansson et al., 1998), compared to for example primary astrocytes (Enkvist et al., 1996) and NG108-15 cells (Holmberg et al., 1998). A reason for this may be promiscuous receptor–G16-protein coupling in HEL cells, which has been shown for purinergic receptors in this cell line (Baltensperger & Porzig, 1997).
Apyrase treatment of CHO cells did not totally abolish, especially in the case of the α_{2B}-AR, the Ca^{2+} response of α_{2}-ARs (IV). In some instances, the Ca^{2+} signalling mediated by α_{2}-ARs is not abolished by Ptx treatment. In Ptx-treated NG108-15 cells, the Ca^{2+} response of the endogenous α_{2B}-AR is inhibited by about 65% (Holmberg et al., 1998). In CHO cells, the α_{2B}-AR-mediated [Ca^{2+}]_i increase is attenuated by about 80% in Ptx-treated cells, whereas α_{2A}- and α_{2C}-AR responses are completely blocked (Kukkonen et al., 1998). Of the α_{2}-ARs, only the α_{2B}-AR subtype mediates a Ca^{2+} response in Sf9 cells, and the response in this cell type is insensitive to Ptx (Holmberg et al., 1998). Contrary to expectations, the Ca^{2+} response of α_{2B}-AR was attenuated by G_{i} coexpression in these cells (Figure 3), suggesting a G_{i/o}-independent pathway. Although the α_{2B}-AR is promiscuously coupled to G_{s}-proteins, the Ca^{2+} response is probably not associated with this G-protein species (I). The hypothesis is that another G-protein than G_{i/o} or G_{s} is involved. A G_{q} subfamily protein is a good candidate. However, we have not detected any change in the Ca^{2+} signalling mode of α_{2B}-AR in Sf9 cells with coexpression of the mammalian G_{11}-protein, which, if coupled to the receptor, would be expected to work in an analogous fashion to G_{s}-protein expression, increasing agonist potency and/or maximum response (III). This does not exclude the possibility of the insect G_{q} as the linker, but additional work will be needed to either exclude or include this, as well as other, potential pathways in α_{2}-AR-mediated Ca^{2+} signalling.
6 SUMMARY AND CONCLUSIONS

The use of BEVS appears well suited for functional characterization of receptor signalling. The signalling components of Sf9 cells are well recognized by mammalian receptors, and heterologous expression of complementary signalling molecules can be used to assess these in signal transduction of particular GPCRs. The lack of endogenous receptors for most mammalian hormones and neurotransmitters in Sf9 cells provides a good cellular background for heterologous receptor characterization. In addition, the predetermined fate of baculovirus-infected cells, with the concomitant inhibited host protein synthesis, can be used advantageously for examination of Ptx-sensitive G-protein function.

The $\alpha_{2B}$-AR was demonstrated to couple promiscuously to $G_s$-proteins both in membrane preparations and live cells. The ability of the mouse and the human $\alpha_{2B}$-AR subtype to induce stimulatory cAMP responses in Sf9 cells through endogenous G-proteins suggests a similarly productive $G_s$ coupling, and an interspecies similarity in this regard. Using a chimeric receptor approach, it was demonstrated that the structure of the i2-loop of $\alpha_{2B}$-AR is a determinant for $G_s$ coupling, and that it is this loop that differentiates the G-protein coupling selectivities of the $\alpha_{2A}$- and $\alpha_{2B}$-ARs. The influence of receptor density on the cellular response is also obvious from the studies. The human $\alpha_{2B}$-AR, expressed at relatively high levels, induces mainly stimulatory responses. The chimeric mouse $\alpha_{2A}/\alpha_{2B}$-i2 loop receptor is also primarily coupled to stimulation at high receptor densities, but exhibits U-shaped inhibitory and stimulatory responses at lower densities. The mouse $\alpha_{2B}$-AR, expressed at relatively low density, exhibits a U-shaped cAMP response.

The AC stimulatory capacity of $\alpha_2$-ARs may not always be associated with a $G_s$-protein coupling, as shown in paper V. The results of paper V demonstrate that a selective activation of $G_i$ or $G_o$ has distinct consequences for AC2 regulation.
Coupling of the $\alpha_{2A}$-AR to $G_o$ resulted in inhibition of the PKC-stimulated AC2 activity. In contrast, $G_i$ activation, by the same receptor, resulted in potentiation of the PKC-stimulated AC2 activity. A $G\beta\gamma$-mediated potentiation of $G_s$-stimulated AC2 activity was evident for both types of G-proteins, indicating that the $\alpha$ subunits of $G_o$ and $G_i$ have different roles for this AC isoform.

$Ca^{2+}$ mobilization by $\alpha_2$-ARs is sensitive to Ptx in some cell types and is likely to be a result of $G\beta\gamma$-mediated stimulation of PLC. In CHO cells, the $Ca^{2+}$ response of $\alpha_2$-ARs was attenuated, almost to zero response in some cases, if extracellular purine nucleotides were removed. This implicated purinergic receptors in the signalling cascade, which was confirmed by attenuation of $\alpha_2$-AR response in the presence of suramin, a purinergic receptor antagonist. The results suggest a receptor cross-talk at the level of PLC enzymes. The tonic purinergic receptor activation will provide PLCs with GTP-loaded $\alpha$ subunits of the $G_q$ subfamily proteins and thereby prime the PLC for $G\beta\gamma$ signalling. Thus, $G\alpha$ subunit activation seems to be obligatory for $G\beta\gamma$ signalling in this model system.

A trend of the $\alpha_{2A}$-AR subtype being promiscuously coupled to $G_s$, whereas the $\alpha_{2B}$-AR is nearly unproductive in $G_s$ coupling, was inferred from the first paper on promiscuous G-protein coupling of $\alpha_2$-ARs (Eason et al., 1992). In our hands, the $\alpha_{2B}$-AR is far more propensive for $G_s$ coupling than the $\alpha_{2A}$-AR. Regarding the $Ca^{2+}$ signalling, all $\alpha_2$-AR subtypes have the ability to regulate PLC activity in CHO cells. In Sf9 cells, only the $\alpha_{2B}$-AR is coupled to a $[Ca^{2+}]_i$ increase, suggesting again a fundamental difference between $\alpha_2$-AR subtypes on functional criteria.
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