A_{2B} adenosine receptor signaling in MDA-MB-231 breast cancer cells: Mechanism of A_{2B}-mediated reduction of ERK1/2 phosphorylation

Signalwege des A_{2B} Adenosinrezeptors in MDA-MB-231 Brustkrebszellen: Mechanismus der A_{2B}-vermittelten Reduktion der ERK1/2 Phosphorylierung

Doctoral thesis for a doctoral degree

at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

Section Biomedicine

submitted by

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Würzburg, 2019

Affidavit

I hereby confirm that my thesis entitled "A_{2B} adenosine receptor signaling in MDA-MB-231 breast cancer cells: Mechanism of A_{2B}-mediated reduction of ERK1/2 phosphorylation" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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"La plus grande gloire n´est pas de ne jamais tomber,
mais de se relever à chaque chute"

Contents

1   Introduction

Adenosine is an endogenous purine ribonucleoside composed of adenine bound to a ribose (Fig. 1) and is present in all mammalian tissues. Most, if not all cells possess equilibrative adenosine transporters (King et al., 2006). Adenosine can pass the plasma membrane through the bi-directional nucleoside transporters (Ballarin et al., 1991; Fredholm et al., 2001; Pastor-Anglada et al., 2018; Young, 2016). For this reason, a finite amount of adenosine will be in the extracellular space, even under basal conditions. In a quiescent cell, the level of adenosine lies in the range of 30-200 nM (Ballarin et al., 1991) and can rise to the low micromolar range under extreme physiological conditions (Pedata et al., 2001; Zetterstrom et al., 1982). Intracellulary, adenosine is produced from its precursor adenosine-5'-monophosphate (5´-AMP) by the action of the enzymes ATP-ase, ADP-ase, and 5'-nucleotidase, and can then follow several metabolic or synthetic pathways. Adenosine can be converted to 5'-AMP by adenosine kinase and subsequently to ADP and ATP by ADPase and ATPase, respectively. Furthermore, adenosine can be metabolized to inosine and hypoxanthine by adenosine deaminase, to uric acid by xanthine oxidase, and to S-adenosylhomocysteine (SAH) by the S-adenosylhomocysteine hydrolase. The concentration of extracellular adenosine increases through synthesis and transport into the extracellular space. In the extracellular space, adenosine is produced through the degradation of ATP to ADP and AMP by ectoenzymes (Ballarin et al., 1991) (Fig. 2). In form of a nucleotide, adenosine plays an important role in energy transfer in various processes of metabolism. Thus, its concentration is dependent on the metabolic state of the cell. The levels of extracellular adenosine vary according to the stress factors present. An increase in energy consumption or a decrease in oxygen supply leads to an enhanced production of adenosine (Linden, 2005). For example, under pathophysiological circumstances, such as in the hypoxic tumor microenvironment or in ischemic areas, extracellular adenosine concentrations can increase by up to 100-fold (30 µM) (Pedata et al., 2001; Spychala, 2000), suggesting that all adenosine receptor subtypes, even the low affinity $A_{2B}AR$, might be activated under these conditions. The possibility must be considered that understanding the diverse adenosine receptor responses in such an environment appears to be indispensable in order to exploit or avoid the receptor mediated signaling to treat diseases.
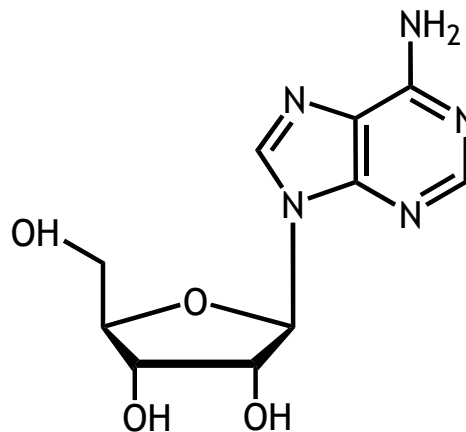
Figure 1:   Structure of adenosine
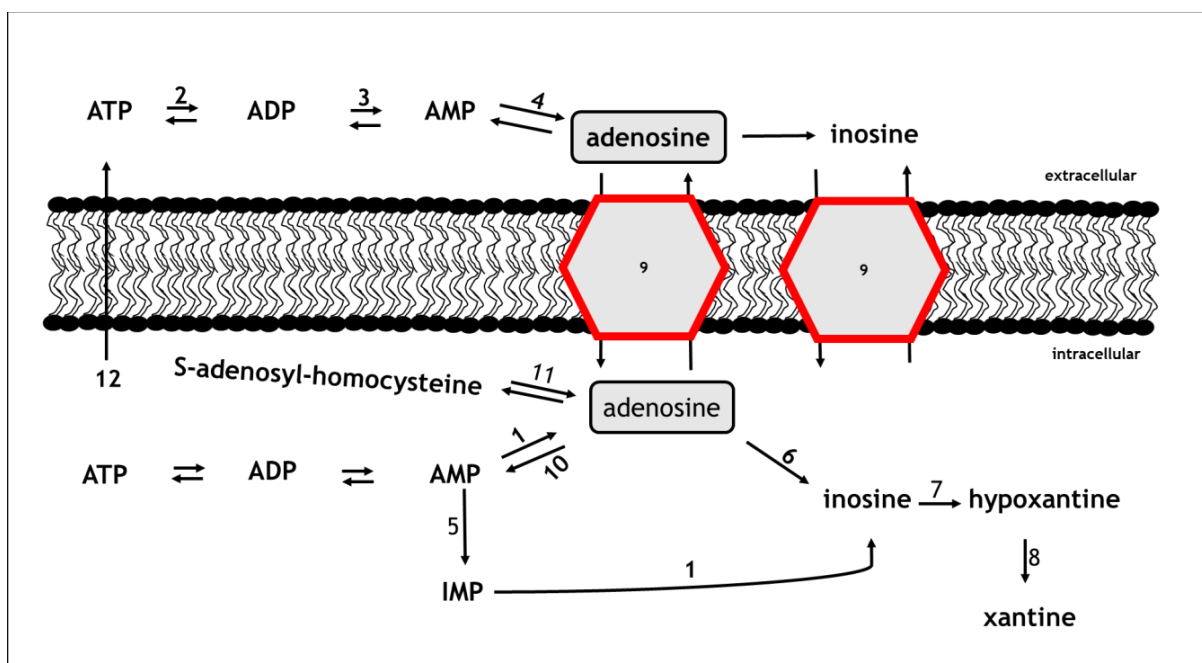
Figure 2:   Adenosine formation and catabolism (modified from Sitkovsky et al., 2004) (1: cytosolic-5´-nucleotidase; 2: ecto-ATPase 3: ecto-ADPase; 4: ecto-5´-nucleotidase; 5: AMP deaminase; 6: adenosine deaminase; 7: purine nucleoside phosphorylase; 8: xanthine oxidase; 9: bidirectional nucleoside transporter; 10: adenosine kinase, 11: SAH-hydrolase, 12: hemichannel)

Maintaining the adenosine balance (Fig. 3) is dependent on the activity and functionality of adenosine deaminase, adenosine kinase, and the nucleotide transporters. Accordingly, hypoxia has been reported to downregulate adenosine kinase activity (Decking et al., 1997). On the other hand, an increase of ATP- and AMP-metabolizing ectoenzyme expression has been shown to increase adenosine

formation (Synnestvedt et al., 2002). Thus, inhibitors of these enzymes or transporters may modulate the adenosine concentration and could be useful in the treatment of certain diseases.
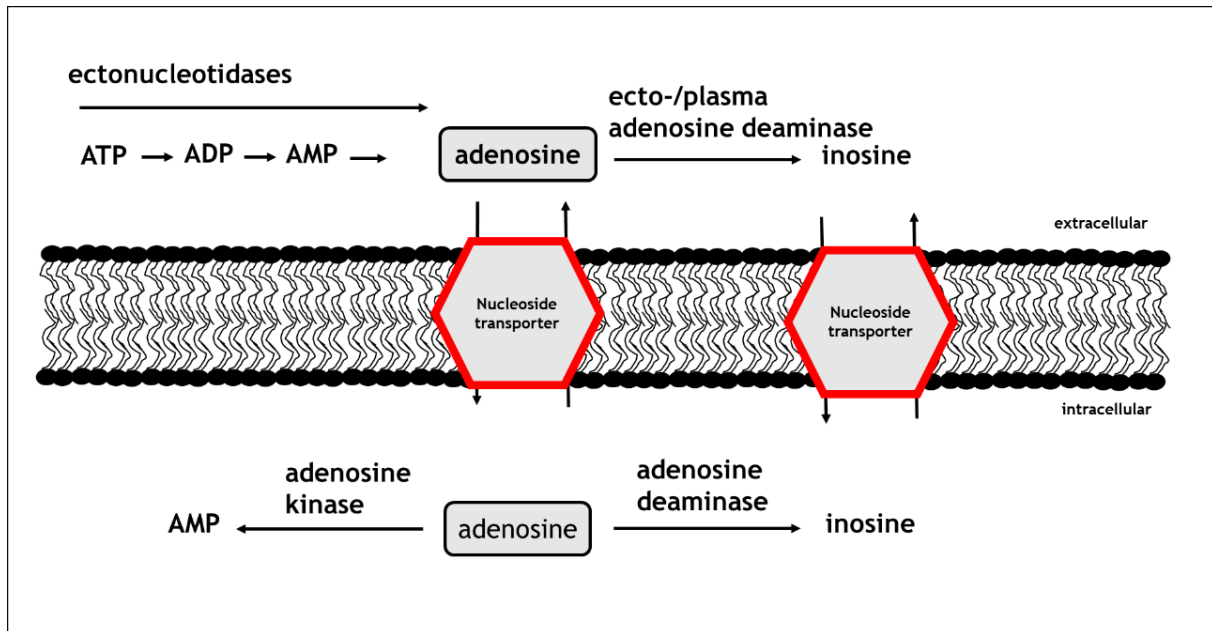
Figure 3: **Intracellular and extracellular balancing system of adenosine (modified from Sachdeva and Gupta, 2013)**

Besides its function as a metabolite, adenosine is involved in the regulation of numerous cellular functions. Drury and Szent-György from the University of Cambridge first observed the effects of adenine nucleotides on cardiac and vascular tissues (Drury and Szent-Gyorgyi, 1929). In 1963, adenosine was characterized as a regulator of coronary vascular tone in response to myocardial hypoxia (Berne, 1963). Subsequently, physiological effects of adenosine in almost all tissues have been observed. In 1970, the increase of cAMP in cerebral slices of guinea pig brain after exposure to different concentrations of adenosine suggested that adenosine could regulate cellular functions via specific adenosine receptors. Based on the responses of various tissues to purines, Burnstock concluded that adenosine and its phosphate derivates mediate their effects through purinergic receptors (Burnstock et al., 1978).

1.1   Purinergic receptors

The purinergic receptors are present in most if not all organ systems in the body. Through the pharmacological analysis of the signal transduction and after molecular cloning, purinergic receptors were divided into two families (Fig. 4): the P1 receptors, also called adenosine receptors (ARs), with adenosine as the main ligand, and P2 receptors, which bind nucleotides such as ATP, ADP, and uracil nucleotides (Ralevic and Burnstock, 1991). The nucleoside monophosphate, AMP, which does not act on any of the P2 receptors, is weakly active as an agonist of ARs. Furthermore, functional expression of adenine receptors, called P0 receptors, has been found (Gorzalka et al., 2005; Knospe et al., 2013).

1.1.1   P2 purinergic receptors

P2 purinergic receptors are divided into two structurally distinct subtypes: the ligand-gated ion channels P2X and the G protein-coupled P2Y receptors (Boeynaems et al., 2005). Seven P2X genes (P2X1-7) have been characterized in eukaryotes. All of them have two intracellular domains and two transmembrane domains connected by a large glycosylated and disulfide-rich extracellular loop. Most P2X purinergic receptors can form homo- and heterotrimers. They are involved in physiological processes as diverse as synaptic transmission, smooth muscle contraction, taste, and inflammation, and serve as targets for the treatment of inflammatory, cardiovascular, and neuronal diseases (Burnstock, 2007). As mentioned above, P2Y receptors belong to the family of G protein-coupled-receptors (GPCR), comprising of an extracellular N-terminus and an intracellular C-terminus, joined by seven trans-membrane domains. The P2Y receptors are activated by nucleotides such as ATP, ADP, UTP, UDP, and UDP-glucose (Jacobson et al., 2011). To this day, eight P2Y receptors subtypes have been characterized. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ couple to G_q/G_{11} to stimulate the phospholipase C (PLC) pathway. While $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ couple exclusively to G_i, $P2Y_{11}$ binds to $G_{q/11}$ and G_i, and therefore modulates both PLC and cAMP signaling pathways. The P2Y receptor-mediated effects have been proposed to be relevant in platelet activation and cystic fibrosis (CF) (Weisman et al., 1998).
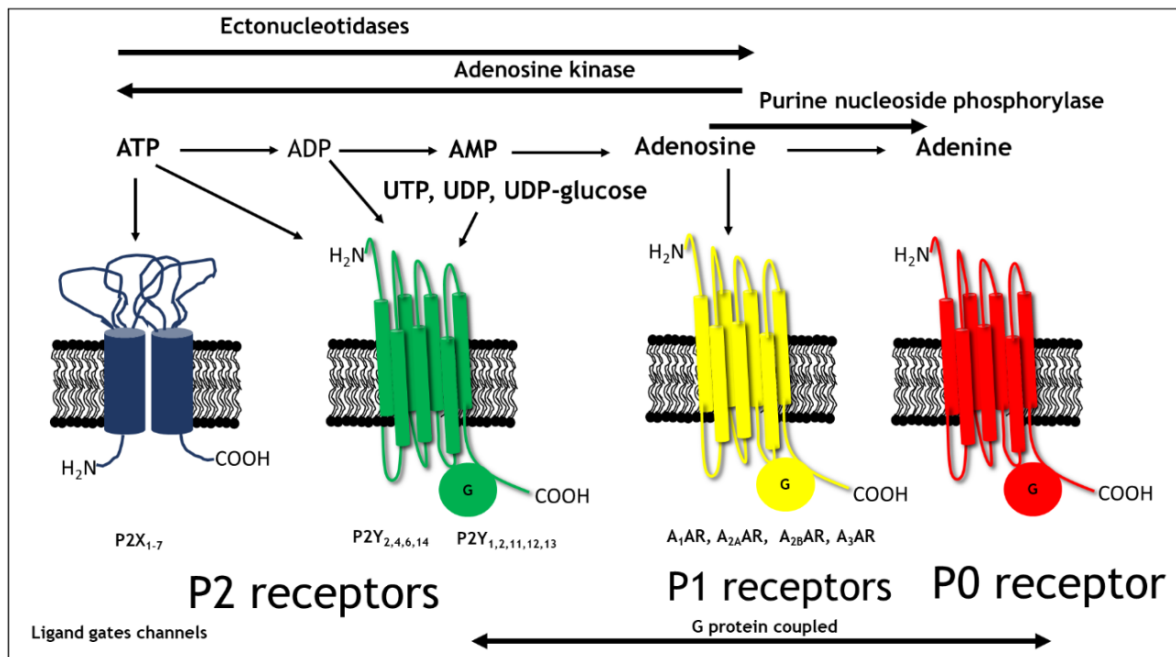
Figure 4: **Purinergic receptor family (modified from Kaebisch et al., 2015)**

1.1.2 P1 purinergic receptors: Adenosine receptors (ARs)

Adenosine mediates most of its physiological and pharmacological effects via stimulation of four G protein-coupled receptors subtypes: A_1ARs, $A_{2A}ARs$, $A_{2B}ARs$, and A_3ARs. The endogenous purine nucleoside adenosine acts as an agonist with a high affinity for the $A_{2A}AR$, A_1AR, and A_3AR receptors (Ki = 700 nM, 310 nM, and 290 nM, respectively) and with lower affinity for the $A_{2B}AR$ (Ki ≥ 10 µM) (Valls et al., 2009). Consequently, A_1ARs, $A_{2A}ARs$, and A_3ARs are classified as high affinity adenosine receptors and $A_{2B}ARs$ as low affinity receptor, thus suggesting that A_1ARs, $A_{2A}ARs$, and $A_{3A}ARs$ adenosine receptors may be the major ARs activated by physiological levels of extracellular adenosine, while $A_{2B}ARs$ become active under pathophysiological conditions (adenosine concentration in µM range). Adenosine receptors are widely expressed. They have a seven transmembrane α-helical structure, with an extracellular amino-domain (N-terminus) and an intracellular carboxy-domain (C-terminus). The N-terminal domain has N-glycosylation sites, which are important for receptor trafficking, while the carboxy-terminus provides phosphorylation sites for protein kinases and is involved in receptor desensitization.

Furthermore, the carboxy-terminus and the third intracellular loop enable coupling of the ARs to G proteins (Sheth et al., 2014) (Fig. 5).
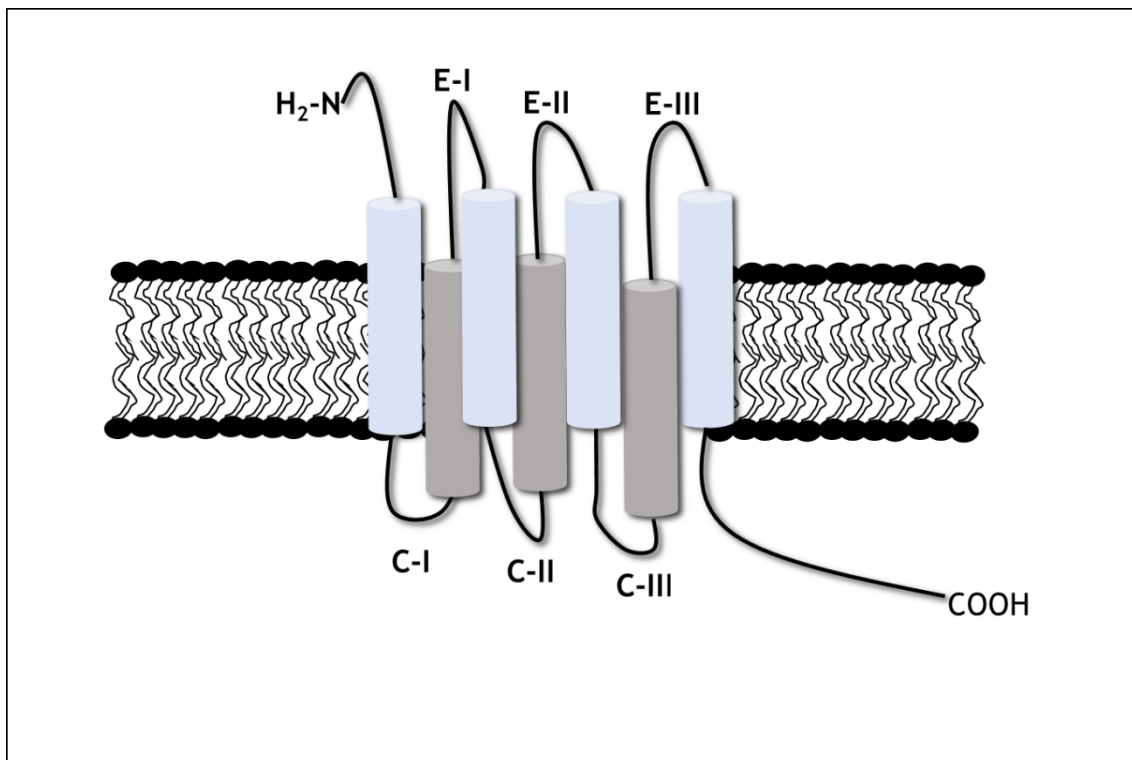
Figure 5: **Adenosine receptor model (modified from Hutchinson and Scammells, 2004)**

Adenosine receptors do not only differ in their affinity for adenosine, but also in the type of G proteins they recruit and in the downstream signaling pathways that are activated (Fig. 6). The A_1ARs and A_3ARs couple to $G_{i/o}$ proteins, leading to inhibition of adenylyl cyclase (AC) and a decreased cAMP production. Consequently, A_1AR and A_3AR stimulation leads to inhibition of PKA activity and subsequent reduction of the phosphorylation of the cyclic AMP response element binding protein (CREB). In contrast, A_{2A}ARs and A_{2B}ARs are linked to $G_{s/olf}$ proteins and therefore stimulate AC activity. Hence, these receptors primarily regulate the production of cyclic adenosine monophosphate (cAMP). However, many studies have shown that adenosine receptors can couple to more than one G protein. For example, A_3ARs interact not only with G_i, but also with G_q and induce, in parallel to AC inhibition, the stimulation of PLC followed by calcium release (Englert et al., 2002). Similary, the A_1AR has been shown to couple to different pertussis toxin-sensitive G proteins

(G_{i1}, G_{i2}, G_{i3}, and G_0) and modulates, in addition to AC, also calcium and potassium channels (Belardinelli et al., 1995) and PLC (Rogel et al., 2005; Tawfik et al., 2005). In addition to G_s, the A_{2A}AR subtype couples to G_{olf} and activates AC and subsequent cAMP production (Kull et al., 2000). The A_{2B}AR, which primarly couples to G_s, also interacts with $G_{q/11}$ (Linden et al., 1999) and stimulates PLC and protein kinase C (PKC) in addition to AC. We have also observed that the endogenous A_{2B}AR in MDA-MB-231 breast cancer cells couples functionally to AC activation and calcium mobilization (Panjehpour et al., 2005), which is in line with other studies. Furthermore, all four AR subtypes have been shown to couple to mitogen-activated protein kinase (MAPK) (Reid et al., 2005; Schulte and Fredholm, 2002, 2003), giving them a role in cell growth, survival, death, and differentiation.
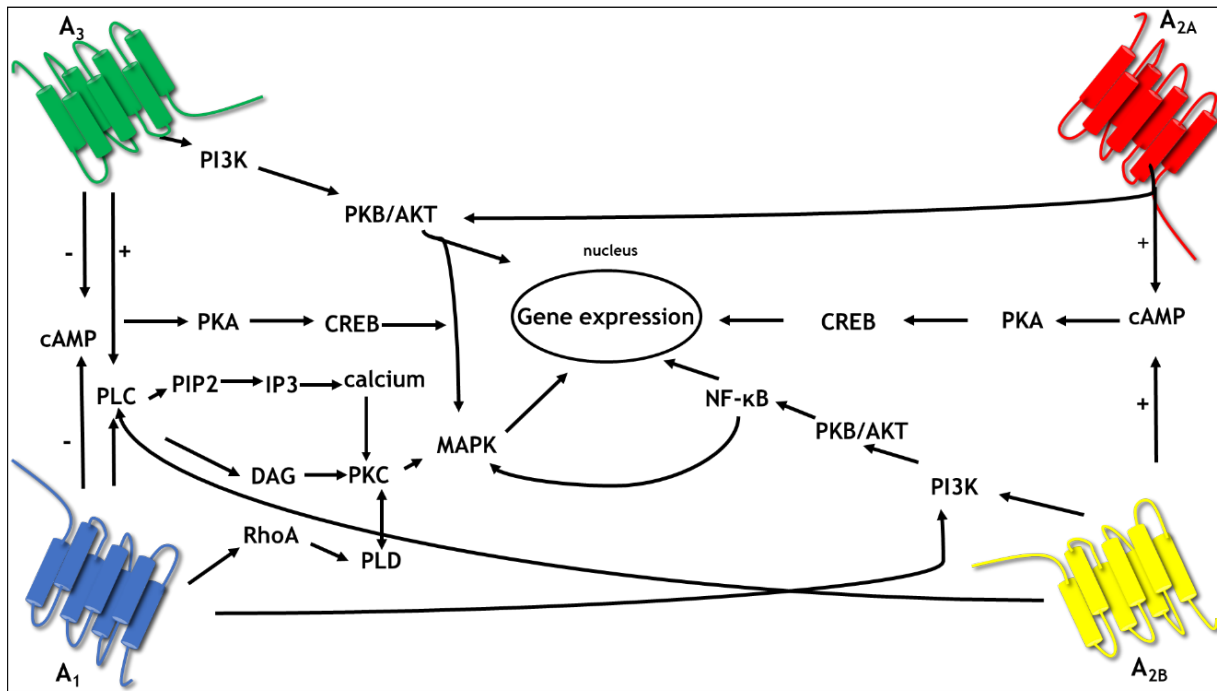
Figure 6: **Adenosine receptor signaling pathways (modified from Jacobson and Gao, 2006)** (Abbreviations: CREB, cAMP response element binding protein; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PK, protein kinase; PLD, phospholipase D; NF-B, nuclear factor-B)

Adenosine receptor subtypes show diverse expression patterns in different tissues and organs, implying the diversity of their physiological and pathophysiological effects. Nevertheless, depending on the effector coupling, the effect might be similar or different. Hence, targeting ARs could provide potent treatment options for many diseases. Through a broad range of screening studies, the potential benefit of drugs targeting ARs has been tested and the results are shown in table 1.

Table1: Diseases targets for selective adenosine receptor ligands (for details see Jacobson and Gao, 2006)

Receptors	Diseases targeted by selective ligands	
	Agonists	Antagonists
A_1AR	Epilepsy, stroke, arrthymia, pain cardiac ischaemia, migrain	Cognitive disease; oedema
$A_{2A}AR$	Sleep disorder, respiratory disorders, thrombosis, sepsis, hypertension, arthritis, kidney ischaemia, inflammation, wound healing, rheumatoid	Parkinston´s disease, Huntingston´s disease; diarrhoea; migraine
$A_{2B}AR$		Asthma, diabetes
A_3AR	Stroke, lung injury, cardiac ischaemia, cancer, rheumatoid, arthritis	Glaucoma asthma renal failure

The list of AR-mediated effects is long and is still increasing. All AR subtypes may be activated in cancer due to increased concentration of adenosine in the hypoxic tumor microenvironment. Addressing the questions of which adenosine receptors are present in cancer tissues and how these receptor subtypes affect cancer growth is quite important. It has been suggested that the A_1AR subtype may act as a potent regulator of normal and tumor cell growth by exerting antiapoptotic and prosurvival effects (Albasanz et al., 2007; Kochanek et al., 2006; Merighi et al., 2003; Morrison et al., 2006; Wendler et al., 2007). The deletion of A_1AR in mice inoculated with Gl261 glioblastoma cells results in an increase in glioblastoma tumor growth (Synowitz et al., 2006). This implies that activation of A_1AR impairs glioblastoma growth. But A_1AR could also contribute to tumor cell growth and survival in breast cancer cells. For example, depletion of A_1AR receptors by siRNA reduces MDA-MB-468 breast cancer cell growth and induces apoptosis (Mirza et al., 2005). A_3AR inhibits

tumor growth in prostate, melanoma, and colon carcinoma cells (Fishman et al., 2003; Fishman et al., 2002; Madi et al., 2003; Madi et al., 2004; Ohana et al., 2003). Under hypoxic conditions, activated A_{2A}ARs mediate immunosuppressive effects, which block antitumor immunity and enable tumor growth (Ohta et al., 2006). Therefore, adding A_{2A}AR antagonists to this type of cancer therapy may enhance the efficacy of tumor immunotherapy. By contrast, activation of A_{2A}ARs could also induce a PKC and MAPKs-dependent apoptotic signaling pathway (Merighi et al., 2002). Among the AR subtypes, the A_{2B}AR requires a higher level of adenosine to be activated because of its low affinity (K_i >10 μM) for adenosine. Consequently, the coexpression of the AR subtypes in the same tissues or cells makes the characterization of the low affine A_{2B}AR in such an environment difficult. Furthermore, several studies revealed an increase in A_{2B}ARs expression in tumor tissue in comparison to normal tissue (Table 2). Of note, the promotor region of A_{2B}AR possesses a functional binding site for hypoxia-inducible factor (HIF) (Eckle et al., 2014) and the concentration of adenosine is increased in response to hypoxia or inflammation such as are present in a solid tumor. While the A_{2B}AR has a low affinity for adenosine, it might be activated during conditions of hypoxia or ischemia when the extracellular adenosine levels rise (Koeppen et al., 2011). Taken together, hypoxic conditions like those present in tumor environments are favorable for the induction and activation of A_{2B}ARs (Feoktistov et al., 2004), which in turn might modulate the tumor growth and progression and influence the outcome of cancer. This suggests a role of A_{2B}AR subtype in mediating adenosine signaling responses under pathological conditions (Eckle et al., 2014; Feoktistov et al., 2004). It is known that A_{2B}AR is important in some pathophysiological conditions, including vascular injury, (Yang et al., 2008) chronic lung disease (Sun et al., 2006), and ischemic disease (Grenz et al., 2008). In addition, the pro-tumoral role of the A_{2B}ARs in cancer cell is well characterized. A_{2B}AR knockout mice infected with Lewis lung carcinoma cells showed a reduction of tumor growth and longer survival in comparison to wild-type mice (Ryzhov et al., 2008), providing the first genetic evidence for a role of A_{2B}AR in tumor progression. Activation of A_{2B}ARs leads to the release of angiogenic factors that could promote tumor growth (Du et al., 2015; Feoktistov et al., 2002; Feoktistov et al., 2004; Rizvi et al., 2013). Furthermore, inhibition of A_{2B}AR impedes the proliferation of colon carcinoma cells (Ma et al., 2010).

Nevertheless, emerging evidence suggest that A_{2B}ARs could also exert an inhibitory effect on tumor cell proliferation (Dubey et al., 2005; Dubey et al., 2001). The A_{2B}AR-mediated antiproliferative effect may be mediated through the reduction of ERK1/2 phosphorylation. This is in line with our observations in MDA-MB-231 cells. We observed a high and unique expression of A_{2B}ARs as the sole adenosine receptor subtype in the MDA-MB-231 breast cancer cells with a link to reduction of ERK1/2 phosphorylation and reduction of proliferation. The diverse pathways that couple to activation of A_{2B}ARs in MDA-MB-231 cells are depicted in Fig. 7. As a consequence, adenosine can be beneficial in certain disorders, and A_{2B}ARs might present potential targets for tumor growth inhibition. However, the inhibition of angiogenesis requires the use of A_{2B}AR antagonists, while inhibition of growth signaling via the MAPK pathway might be achieved through A_{2B}AR agonists in MDA-MB-231 cells. Understanding how A_{2B}AR modulates both effects may help in therapeutic intervention in some types of diseases.

Table 2: A_{2B}ARs expression in tumor tissue in comparison to normal tissue

Cancer Type	Sample	A_{2B}AR expression relative to normal tissue	References
Breast cancer	Breast cancer datasets (n = 5715)	High expression (up to 40% possibilities for worse outcome)	Cancer Res., 76 (2016), pp. 4372-4382
Prostate	Prostate cancer and adjacent non-cancerous samples from 20 patients	2.4 times increase in respect to normal tissue	Prostate, 75 (2015), pp. 735-747

Epithelial ovarian cancer	15 primary cultures of normal ovarian surface epithelial cells and 17 primary cultures of malignant ovarian tumor	9.7 times decrease respect to normal tissue	Int. J. Gynecol. Cancer, 18 (2008), pp. 963-975
Colorestal cancer	Colorectal cancer and normal colon tissues from 5 patients	8 - 10 times increase respect to normal tissue	Hum. Pathol., 41 (2010), pp. 1550-1557
Hepatocellular cancer	Samples from 64 patients with HCC and adjacent normal tissue	3 times increase respect to normal tissue	Hepatol. Res., 36 (2006), pp. 56-60
Squamous cell carcinoma of lung	10 samples from patients with lung squamous cell carcinoma and normal lung tissue	2.09 times increase respect to normal tissue	Dis. Markers, 24 (2008), pp. 41-50

1.2 Signal transduction via A$_{2B}$AR in MDA-MB-231 cells

The MDA-MB-231 cell line is an epithelial human breast cancer cell line that was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma. MDA-MB-231 is a triple negative breast cancer (TNBC) cell line as it lacks oestrogen receptor (ER), progesterone receptor (PR), as well as HER2 (human epidermal growth factor receptor 2) expression. It represents one of the most commonly used breast cancer cell lines in medical research laboratories. Panjehpour et al., (2005) have shown that the MDA-MB-231 cell expresses a high level of A$_{2B}$AR as the sole adenosine receptor subtype. Using diverse biochemical tools, they showed that A$_{2B}$AR activation stimulates both AC and PLC, which leads to cAMP production and an increase in intracellular calcium, respectively. Furthermore, a high basal phosphorylation of extracellular signal-regulated kinase (ERK1/2) was observed in these cells (Koussémou et al., 2018). The

incubation of MDA-MB-231 cells in presence of the nonselective adenosine receptor agonist NECA leads to a reduction of ERK1/2 phosphorylation (Fig. 7).
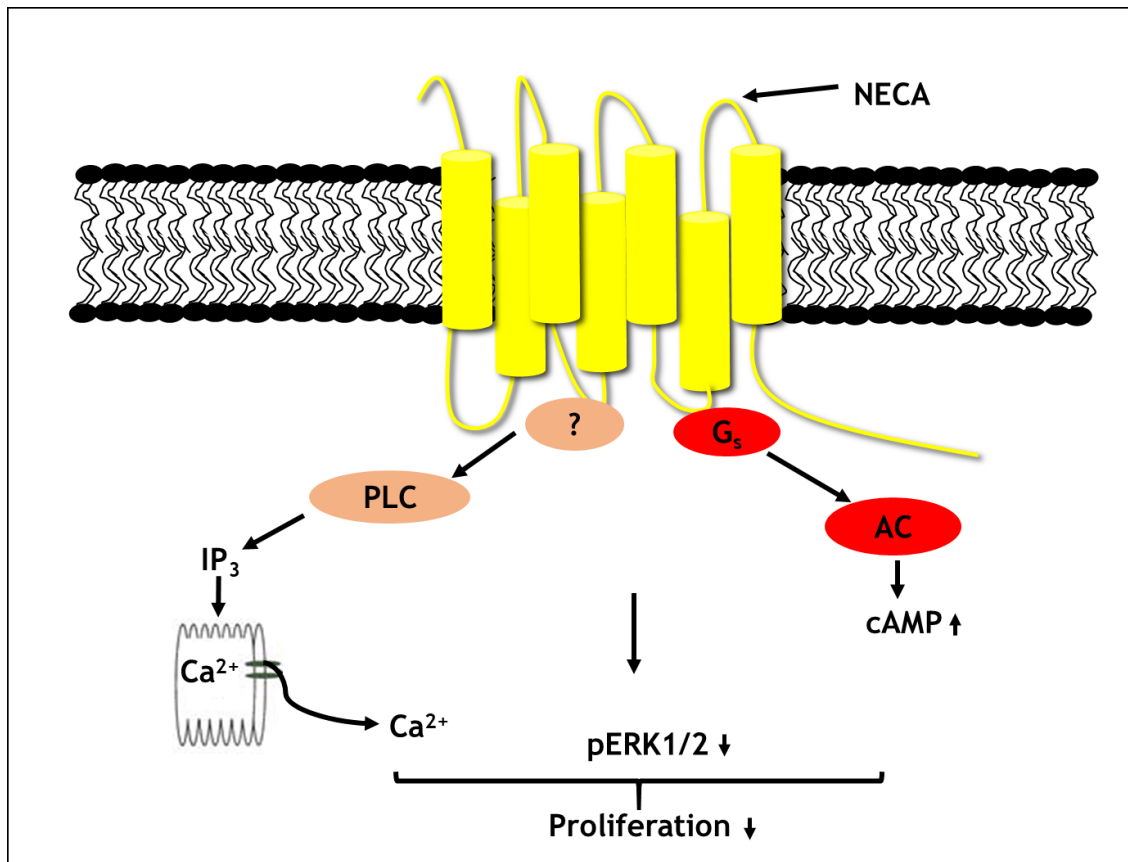
Figure 7: **A$_{2B}$AR-mediated pathways in MDA-MB-231 cell**

MAPKs are widely expressed and are activated by diverse stimuli, including G protein–coupled receptors. Activated MAPKs regulate many cellular processes (Kyriakis and Avruch, 2001; Pearson et al., 2001), like cell proliferation and differentiation (Raman et al., 2007). Recent studies have shed some light on the effect of adenosine receptors on the MAPK pathways. Depending on the cell type, adenosine receptors can modulate the activation or inhibition of MAPK signaling pathways. ERK1/2 belongs to the MAPKs, and all four AR subtypes are coupled to ERK1/2 phosphorylation in transfected Chinese hamster ovary (CHO) cells (Schulte and Fredholm, 2000, 2002, 2003). Furthermore, we previously observed in our laboratory a decrease in ERK1/2 phosphorylation in MDA-MB-231 cells after stimulation with the nonselective adenosine receptor agonist NECA (Koussémou et al., 2018). Therefore, it is suggested that activation of A$_{2B}$AR may negatively regulate the development of

tumors via reduction of the ERK1/2 phosphorylation. In contrast, activation of the $A_{2B}AR$ plays a role in the development of tumors via upregulation of the expression levels of angiogenic factors. The MAPK pathways are critically important in the regulation of cell proliferation and differentiation and have been the subject of intense research leading to the development of pharmacological inhibitors for the treatment of cancer. Nevertheless, the role of $A_{2B}AR$ in human disease therapy, more specifically in cancer therapy, remains a major knowledge gap. A better characterization of how the $A_{2B}AR$ subtype participates in the molecular mechanisms underlying reduction of ERK1/2 phosphorylation and inhibition of cell proliferation will lead to substantial improvements in the clinical management of various cancers.

1.3   Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases are evolutionarily conserved enzymes present in all eukaryotes and they represent a central regulatory network (Manning et al., 2002). Mammalian MAPKs comprise three major subgroups that are classified based on sequence similarity, differential activation, and substrate specificity. These include the extracellular signal-regulated protein kinases (ERKs), the c-Jun amino-terminal kinases (JNK1-3) and the p38 proteins (α, β, γ, and δ) (Cuadrado and Nebreda, 2010; Garrington and Johnson, 1999; Johnson and Lapadat, 2002; Roskoski, 2012a; Weston and Davis, 2002). Most studies have shown that the ERK1/2 pathway can be induced by a variety of factors and thereby regulate growth, proliferation, and anti-apoptotic signaling cascades (Johnson and Lapadat, 2002; Roskoski, 2012a). The JNK and p38 pathways are primarily induced by numerous forms of stress-related stimuli and therefore classified as stress-activated protein kinases (SAPKs), but they also respond to stress independent stimuli (Coulthard et al., 2009; Cuadrado and Nebreda, 2010; Davis, 1994; Johnson and Nakamura, 2007; Kyriakis and Avruch, 1996, 2001). A schematic representation of the signal cascades is shown in Fig. 8. The MAPK activation is transmitted through a three-component protein kinase cascade consisting of the serine threonine MAPK kinase kinase (MAPKKK), which phosphorylates and activates a dual-specificity protein kinase, MAPK kinase (MAPKK), which in turn phosphorylates and activates another serine threonine protein kinase MAPK. The cascade results in the dual phosphorylation of a tyrosine and a threonine within the TXY motif of the corresponding MAPK. All MAPKs are activated by specific

MAPKKS. But each MAPKK can be activated by more than one MAPKKK, which in turn respond to various stimuli, increasing the diversity and complexity of MAPK signaling. Activation of ERK1/2 depends on the MAPKKK c-Raf-1 (Chang and Karin, 2001). In contrast, JNK and p38 cascades respond to many stimuli and can be activated by a dozen MAPKKKs. Once activated, these MAPKs in turn activate diverse effectors by phosphorylation influencing many cellular processes, like growth, differentiation, proliferation, and programmed cell death (Kyriakis and Avruch, 2001; Pearson et al., 2001). One of the most explored functions of MAPK signaling is the regulation of gene expression by direct or indirect phosphorylation and subsequent activation of transcription factors (TFs) (Whitmarsh, 2007). Because of their numerous downstream targets, the MAPKs influence diverse metabolic and signal transduction pathways. Thus, the role of MAPK cascades in human diseases therapy, more specifically in cancer therapy, remains a major knowledge gap. Consequently, the modulation of the magnitude, duration and fine tuning of MAPK activities are important for the biological outcome (Ebisuya et al., 2005). The regulation of the kinase catalytic activity requires among others the activity of phosphatases. Understanding how the MAPKs and phosphatases worked together to modulate diverse cellular processes will provide valuable information for diverse therapies.
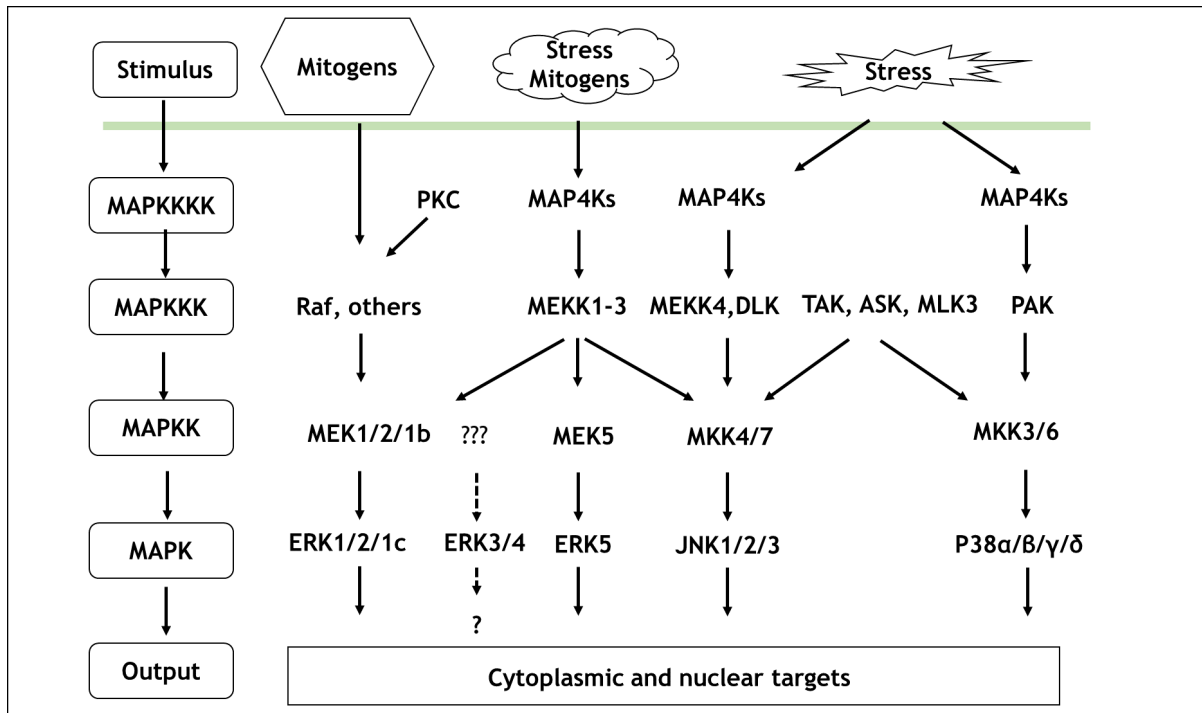
Figure 8: Schematic representation of MAPK cascades (modified from Garrington and Johnson, 1999; Plotnikov et al., 2011).

1.4 Dual-specificity phosphatases (MKPs)

Activated MAPKs have been shown to phosphorylate numerous downstream effectors and subsequently activate many signaling pathways, including cell proliferation and apoptosis. Thus, inhibition of MAPK might offer an attractive treatment strategy in a variety of diseases. Because phosphorylation activates MAPKs, dephosphorylation has been proposed to control the intensity and the duration, i.e. the fine tuning of the MAPK signal. This can be achieved by tyrosine-specific phosphatases, serine-threonine phosphatases or dual-specificity (Thr/Tyr) phosphatases (Keyse, 2000). However, in mammalian cells deactivation of MAPKs is primarily carried out by the dual specificity protein phosphatases often referred to as MAPK phosphatases (MKPs). MKPs are a family of dual-specificity protein phosphatases. Eleven members have been identified, which differ from each other in their expression pattern and expression kinetics (Dickinson and Keyse, 2006). Based on sequence similarity, gene structure, substrate specificity, and subcellular localization, the MKPs were further divided into three groups (Fig. 9). Most MKP genes are silent in resting cells, but highly inducible in response to growth factors and stress stimuli. The serine/threonine and tyrosine-specific phosphatases control the activity of MAPKs during an initial phase in the cytosol. However, activated MAPKs that eventually

translocate to the nucleus, phosphorylate TFs and induce immediate early genes including those coding for dual specificity MKPs. Once translated, MKPs completely abrogate signaling by dephosphorylating MAPKs. Among them, MKP-1 and MKP-2 recognize ERK2, JNK2, and p38, ERK2 and JNK2, respectively (Chu et al., 1996). The MKPs expression is controlled at transcriptional, post-transcriptional, and post-translational levels (Moosavi et al., 2017). MKPs act in different cellular compartments and may control not only the amplitude and duration but also the localization of MAPK signals, making the outcomes of cellular response complex but more specific.
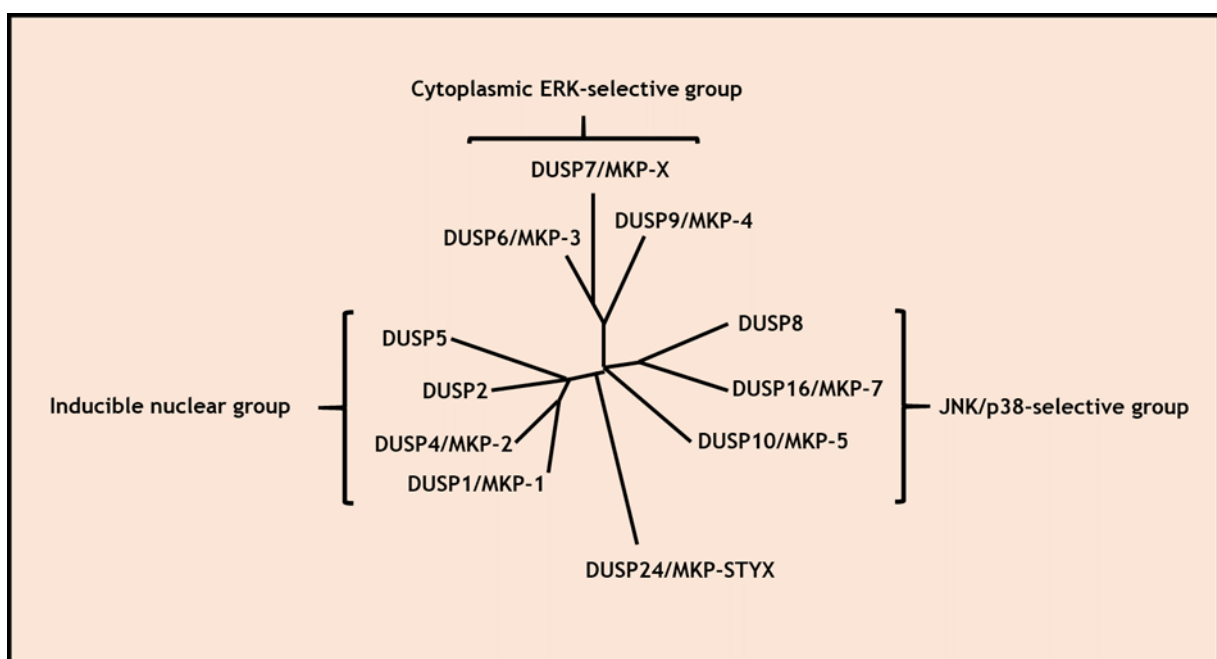
Figure 9: **DUSP phylogenetic tree, for details see (modified from Dickinson and Keyse, 2006)**

1.5 Functional selectivety of A$_{2B}$AR ligands

Historically, GPCRs were believed to function as binary switches, and could only exist in two conformations, either in the active or in the inactive state. According to this concept, activation or inhibition of GPCRs would equally affect all downstream signals. This simplified definition can no longer be maintained nowadays. Receptors are capable of much more than producing simple binary signals. Some can signal through different G-proteins or through multifunctional adapters, β-arrestins. Thus, GPCRs exist in multiple conformational states, with each conformation leading to

different downstream effects, a concept referred to as functional selectivity (Kenakin, 2001, 2005; Urban et al., 2007). Consequently, different ligands may differentially activate intracellular signaling pathways and this concept is termed biased agonism (Kenakin and Miller, 2010). In particular, the concept of ligand bias, whereby a ligand can induce different receptor conformations leading to activation of multiple signaling pathways with differing efficacies (Kenakin, 2001), gained increasing attention. For example, a full agonist for one dictinct GPCR-mediated response might be an antagonist or inverse agonist for another signal elicited by the same receptor. Thus, different agonists might be able to preferably trigger the interaction of the receptor with distinct signaling pathways. Likewise, in the classic concept of pharmacology, antagonists prevent or alter the binding of agonists to the receptor. Competitive receptor antagonists bind to the agonist binding site and prevent receptor activation by precluding agonist binding. Non-competitive antagonists bind to an allosteric site, causing a conformational change in the receptor, which will preclude the binding of an agonist. Furthermore, antagonists do not function only as inhibitors of agonist-induced function but can also provoke endogenous signals (Azzi et al., 2003; Baker et al., 2003; Costa and Herz, 1989). Despite their capacity to mediate therapeutic effects, most drugs exhibit undesirable effects to various degrees (Violin and Lefkowitz, 2007). In comparison to conventional balanced ligands, biased ligands seem to have different functions and physiological consequences (Rajagopal et al., 2010). They offer the possibility to selectively induce favorable signaling with limited adverse reactions. Consequently, biased ligands may offer a therapeutic benefit, by limiting undesired effects (Urban et al., 2007). One can note that identification of biased ligands may offer the possibilty to selectively modulate the diverse $A_{2B}AR$-mediated signaling pathways to deliver precise therapeutic benefit, and at the same time minimize undesired effects.

2   Specific Aims

Despite growing progress in cancer therapy, triple negative (ER-, PR-, HER2-negative) breast cancer receives less attention than other types of cancer. Triple negative breast cancer occurs in about 10-20% of diagnosed breast cancers and is more likely to affect young people. Triple negative breat cancer patients do not respond to common treatment like hormone therapy. Drugs that target estrogen, progesterone, and HER2 receptors are ineffective. Using chemotherapy to treat triple negative breast cancer is an option, but the prognosis is influenced by the stage of the cancer and the grade of the tumor. Despite the lower number of triple negative breast cancer patients, it is important to search for alternative treatment to improve patient survival.

MDA-MB-231 is a triple negative breast cancer cell line because of the lack of estrogen and progesterone receptors as well as human epidermal growth factor receptor 2 (HERB-2) expression. Recently, it was shown that MDA-MB-231 breast cancer cells express very high levels of the $A_{2B}AR$ as the sole adenosine receptor subtype. Of note, stimulation of the $A_{2B}AR$ in MDA-MB-231 cells triggers an unusual inhibitory signal on ERK1/2 phosphorylation in addition to AC activation and calcium mobilization. The ERK1/2 pathway is reported to be associated with the control of growth, proliferation and differentiation of cells and as such might serve as a promising target for tumor treatment. Thus, understanding how $A_{2B}AR$ activation reduces the ERK1/2 phosphorylation in MDA-MB-231 cancer cell lines may help to develop a new approach for treating triple negative breast cancer. Therefore, the specific aim was to identify and understand in detail the signaling pathways responsible for the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation.

Like most GPCRs, $A_{2B}ARs$ couple to multiple signaling pathways and can thus mediate both beneficial and undesirable effects, depending on the activated pathway. Hence, the search for ERK1/2-biased ligands showing no or low activity on AC- and Ca^{2+}-pathways is of great importance for selective activation of $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation. Another specific aim was therefore to identify potential ERK1/2-biased ligands in MDA-MB-231 cells. For this purpose, we selected the structuraly diverse adenosine-derived nucleoside agonists NECA, PHPNECA, DPA23, UK-432097, and LUF6210 as a nonnucleoside agonist and investigated their

functional activity in AC activation, calcium mobilization, and reduction of ERK1/2 phosphorylation. We hypothesized that the diverse agonists would show, in comparison to the reference ligand NECA, different efficacies when signaling to different intracellular pathways.

In addition, we also analyzed potentially selective effects of diverse antagonists on the A_{2B}AR mediated responses. We chose the nonselective antagonist EFA [ANR 152], the A_1/A_{2B} antagonist DPCPX, and ZM241385, which shows high affinity for A_{2A}AR but also reasonable affinity for A_{2B}AR. Overall, the pharmacological profiles of the tested agonists and antagonists were established for all tested functional responses.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and Products

Name	Supplier
[γ-^{32}P]-Adenosine triphosphat	Hartmann Analytik
Adenosine deaminase (ADA)	Sigma Aldrich
3-Aminophthalhydrazide (Luminol)	Sigma Aldrich
Ammonium persulfate (APS)	Merck
Adenosine triphosphate (ATP)	Sigma Aldrich
Bovine serum albumin (BSA)	Applichem
Bromophenol Blue	Applichem
Calcium chloride	Merck
cAMP	Sigma Aldrich
Creatine kinase (CK)	Roche
Creatine phosphate (CP)	Roche
p-Coumaric acid	Roth
Developer and Replenisher	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Applichem
1,4 Dithiothreitol (DTT)	Sigma Aldrich
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Roth
Ethyleneglycol-bis(ß-aminoethyl) -N,N,N',N'-tetraacetic acid (EGTA)	Merck

Fetal calf serum (FCS)	Biochrom
Fixer and Replenisher	Sigma Aldrich
Fuji – x-ray film Super RX-N	Hartenstein
Fura-2-acetomethyl ester (Fura-2AM)	Invitrogen
Glycine	Applichem
Guanosine triphosphate (GTP)	Sigma Aldrich
HEPES	Sigma Aldrich
Hydrocloric acid fuming 37%	Merck
Hydrogen peroxide (H_2O_2)	Sigma Aldrich
Immobilon-P PVDF-Membrane	Merck
Isoproterenol hydrochloride	Sigma Aldrich
Kaliumchloride (KCl)	Applichem
Lipofectamine 2000	Thermo scientific
Magnesium chloride ($MgCl_2$)	Merck
Methanol	Sigma Aldrich
Di-sodiumhydrogenphophat (Na_2HPO_4)	Applichem
Nonfat dried milk powder	Applichem
Nonidet P-40	Applichem
Opti-MEM-Reduced-Serum Medium	Thermo scientific
Penicillin (100 U/ml)	Gibco-Life Technologies
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Aldrich
Pierce^{TM} Prestained Protein MW Marker	Thermo scientific

Potassium phosphate monobasic	Sigma Aldrich
Rotiphorese® Gel 30 (37,5:1)	Roth
Scintillation LSC-Universalcocktail	PerkinElmer
Scintillation Tubes	Packard
Sodium azide (NaN_3)	Merck
Sodium bicarbonate ($NaHCO_3$)	Applichem
Sodium carbonate (Na_2CO_3)	Merck
Sodium chloride (NaCl)	Applichem
Sodium dodecylsulfate (SDS)	Applichem
Sodium fluoride (NaF)	Merck
Sodium orthovanadate	Sigma Aldrich
Sodium pyrophosphate ($Na_4P_2O_7$)	Sigma Aldrich
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Applichem
Tris(hydroxymethyl)aminomethane	Applichem
Triton™ X-100	Applichem
Trypsin inhibitor from Soybean	Sigma Aldrich
Reaction tubes	Sarstedt
Tween-20	Applichem
Whatman gel blotting papers	Sigma Aldrich
Zinc acetate dihydrate (ZnAC)	Merck
β-Mercaptoethanol	Applichem

3.1.2 List of inhibitors and activators

Name	Effector	Supplier
8-Br-2´-O-Me-cAMP-AM	EPAC activator	Tocris
BAPTA-AM	Cell permeable selective Ca^{2+} chelator	Abcam
cAMP-AM	Cell pemeable cAMP analog	Biolog
CCPA	A_1AR agonist	Prof. Cristalli, University of Camerino, Italy
CGS 21680	$A_{2A}AR$ agonist	Biotrend
Cycloheximide	Proteine synthesis inhibitor	Sigma Aldrich
DPA23	$A_{2B}AR$ agonist	Prof. Baraldi, University of Ferrara, Italy
DPCPX	$A_1AR/A_{2B}AR$ antagonist	Tocris
EFA (ANR 152)	Nonselective antagonist	Prof. Volpini, University of Camerino, Italy
Forskolin	AC activator	Tocris
GF109203X	PKC inhibitor	Tocris
H89	PKA inhibitor	Santa Cruz
HEMADO	A_3AR agonist	Sigma Aldrich
KT5720	PKA inhibitor	Sigma Aldrich
LUF 6210	$A_{2B}AR$ agonist	Prof. AP IJzerman,University of Leiden, The Netherlands

MRS 1220	$A_{3A}AR$ antagonist	Tocris
NECA	Nonselective ARs agonist	Sigma Aldrich
PHPNECA	Nonselective ARs agonist	Prof. Cristalli, University of Camerino, Italy
PMA	PKC activator	Abcam
RO-20-1724	PDE4 inhibitor	BIOMOL
Sanguinarine	MKP-1 inhibitor	Tocris
SB 202190	p38 inhibitor	Sigma Aldrich
SCH 58261	$A_{2A}AR$ antagonist	Prof. Baraldi, University of Ferrara, Italy
U73122	Phospholipase C inhibitor	Tocris
U73343	Inactive analog of U73122	Calbiochem
UK-432097	$A_{2A}AR$ agonist	Pfizer
UTP	$P2Y_2$ agonist	Sigma Aldrich
ZM241385	$A_{2A}AR$ antagonist	Tocris

3.1.3 Oligonucleotides

siRNA	Target	Supplier
MKP-1 siRNA (sc-35937)	MKP-1	Santa Cruz
On-target plus human DUSP4 (1846) siRNA-smart pool (L-003963-00-0005)	MKP-2	Dharmacon
Control siRNA-A (sc-37007)	Non target	Santa Cruz

3.1.4 Antibodies

The following antibodies were used as primary antibodies in immunoblot.

Primary antibodies		
Antibody	**Description**	**Supplier**
ERK1/2	Rabbit, polyclonal, recognizes ERK1 and ERK2	Cell Signaling
Phospho-ERK1/2	Rabbit, polyclonal, recognizes phospho-ERK1/2 (Thr202/Tyr204)	Cell Signaling
MKP-1 (C-19)	Rabbit polyclonal recognizes MKP-1/DUSP-1	Santa Cruz
DUSP4/MKP2	Rabbit, monoclonal, recognizes endogenous MKP-2/DUSP4	Cell Signaling
Phospho-DUSP-1/MKP-1	Rabbit, monoclonal, detects phospho-DUSP1/MKP1 (Ser359)	Cell Signaling
Phospho-JNK	Rabbit, polyclonal, recognizes phospho-JNK (Thr183/Tyr185)	Cell Signaling
Phospho-p38	Rabbit, polyclonal, recognizes phospho-p38 (Thr-180/Tyr-182)	Cell Signaling
Phospho-MEK1/2	Rabbit, polyclonal, recognizes phospho-MEK1/2 (Ser217/221)	Cell Signaling
Phospho-c-Raf (S338)	Rabbit, monoclonal, detects endogenous levels of phospho-c-Raf (S338)	Cell Signaling

Phospho-c-Raf (S259)	Rabbit, monoclonal, detects endogenous levels of phospho-c-Raf (S259)	Cell Signaling
GAPDH	Mouse, monoclonal, detects GAPDH	Santa Cruz
anti-β-tubulin	Mouse, monoclonal, detects β-tubulin	Sigma

For immunoblot analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) were utilized.

Secondary antibodies	
Antibody	**Supplier**
Goat anti-mouse-HRP	Dianosa
Goat anti-rabbit-HRP	Dianosa

3.1.5   Cell lines

CHO-A_{2B}AR cells were available in our group. The MDA-MB-231 and HEK 293 cell lines were obtained from Universitätsklinik Essen, Germany and ATCC, USA, respectively, and were cultivated according to the supplier's recommendations.

Cell line	Description	Reference
MDA-MB-231	ER-negative breast cancer cells	Institute of Cell Biology (Tumor Research), University clinic, Essen, Germany
CHO-A_{2B}AR	CHO cells stably transfected with human A_{2B}ARs	AG Prof. Klotz, University of Würzburg, Germany
HEK 293T	Human embryonic kidney fibroblasts transformed with adenovirus Typ V DNA	ATCC, Manassas, VA, USA

3.1.5   Cell culture media

The following Gibco media and additives were obtained from PAN-Biotech GmbH, Aidenbach:

1. Dulbecco´s phosphate buffered saline

2. Dulbecco´s modified eagle medium (DMEM) with 4.5 mg/ml glucose without L-Glutamine

3. Opti-MEM reduced serum medium

4. 200 mM L-Glutamine and 10,000 U/ml Penicillin /streptomycin

Media were supplemented with heat-inactivated fetal calf serum (FCS), L-glutamine, penicillin and streptomycin according to the requirements of each cell line.

3.1.6   Stock solutions and commonly use buffers

Name	Recipe		
Membrane preparation and adenylyl cyclase activity buffers			
REA-MIX (5 ×)	10 mM cAMP 1 % BSA 1 mM GTP 10 mM ATP 100 mM MgCl$_2$ 1000 mM Tris-HCl, pH 7.4 100 mM RO 20-1724		
Incubation REA-MIX (1ml)	0.4 ml REA-MIX (5 ×) 5.9 mg CP 0.8 mg CK 3,000,000 counts [a-^{32}P] ATP		
Incubation medium	**Agonists** 20 µl 30 µl 50 µl	**Antagonists** 10 µl H$_2$O 10 µl, 5 µM NECA 30 µl membranes 50 µl Incubation REA-MIX	

Western blot stock solution	
PBS	137 mM NaCl 27 mM KCl 80.9 mM Na_2HPO_4 1.5 mM KH_2PO_4, pH 7.4
10 × Phophatase inhibitor mix	500 mM NaF 50 mM $Na_4P_2O_7$ 10 mM Na_3VO_4 3 mM NaN_3 50 mM β-Glycerolphosphat
4 × Laemmli	0.2 M Tris-HCl, pH 6.8 8 % (m/v) SDS 40 % (m/v) Glycerol 0.05 % (m/v) Bromophenol blue 20 % (v/v) β-Mercaptoethanol
Protease inhibitor (PI)	0.1 % Trypsin inhibitor 25 mM Benzamidine 50 mM Tris-HCl, pH 7.4
100 × PMSF	100 M PMSF in 100 % Ethanol
Western blot sample preparation	
Lysis buffer (electrophoresis sample buffer)	0.65 M PBS 1 × 4 × Laemmli 1 × 10 × Phophatase inhibitor mix 1 × 10 × Protease inhibitor 1 M PMSF
SDS-Page gels buffers and preparation	
4 × Lower gel buffer	1.5 M Tris-HCl, pH 8.8 0.4 % SDS
4 × Upper gel buffer	0.5 M Tris-HCl, pH 6.80 4 % SDS

10 × Running buffer	0.25 M Tris-Base 1.92 M Glycine 1 % SDS
APS	10 % $(NH_4)_2S_2O_8$ in H_2O
Western blot protein transfer and detection	
Transfer buffer	20 mM Tris-HCl 150 mM Glycine 20 % Methanol
Western blot blocking Milk	100 mM NaCl 5 % Milk powder 10 mM Tris-HCl, pH 7.4 0.1 % Tween.20
Western blot blocking BSA buffer	150.0 mM NaCl 3.0 % BSA 20.0 mM Tris-HCl, pH 7.4 1 % Tween.20 0
Western blot washing BSA buffer	150 mM NaCl 0.25 % BSA 50.0 mM Tris, pH 7.4 0.25 % NP40
Stripping buffer	100 mM Glycin, pH 2.5 0.1 % SDS
10 × TBS (1 L)	0.2 M Tris, pH 7.6 1.4 M NaCl
Protein detection	
Solution A	90 mM p-coumaric acid 250 mM Luminol 100 mM Tris, pH 8.8
Solution B	18 % H_2O_2 (Perhydrol 30 %) 100 mM Tris-HCl, pH 8.8

Ca^{2+}-assay buffer	
Ca^{2+} loading buffer (CLB)	118 mM NaCl
	5 mM KCl
	1 mM CaCl$_2$
	1 mM MgCl$_2$
	5 mM Glucose
	15 mM HEPES, pH 7.3

3.2 Methods

3.2.1 Cell culture

MDA-MB-231 breast cancer cells were grown adherently and maintained at 37°C in DMEM containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) in 5% CO$_2$/95% air. Cells were split 2 or 3 times weekly at a ratio between 1:4 to 1:8 (Panjehpour et al., 2005). Fetal calf serum was omitted on the day of the experiment.

3.2.2 [^{3}H]-thymidine incorporation assay

Proliferation of MDA-MB-231 cells in response to NECA application was determined by [^{3}H]-thymidine incorporation as described in Schmid et al., (2015). Cells were seeded in 24-well plates for 24 hours and then labeled with [^{3}H]-thymidine (0.5 µCi/ml) in medium supplemented without or with NECA (10 mM). After 12 or 24 hours as indicated, plates were washed twice with PBS, followed by 5% (w/v) trichloroacetic acid (TCA). TCA-precipitated proteins and DNA were lysed in NaOH (0.1 M) and incorporated [^{3}H]-thymidine was quantified by scintillation counting.

3.2.3 Membrane preparation and adenylyl cyclase activity

The membranes for the measurement of adenylyl cyclase activity were prepared from fresh cells on the day of the experiment as described in Klotz et al., (1998). Confluent MDA-MB-231 cells were washed with PBS, collected in ice-cold hypotonic

buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized on ice using the homogenizer ultra turrax T-25 at 24000 min^{-1} for 5 seconds, 2 times. The crude membrane fraction was sedimented from the supernatant at 4°C with the Ti70 rotor at 27,000 rpm for 25 min. The resulting pellet was resuspended in 50 mM Tris, pH 7.4 (Klotz et al., 1998) und ADA was added for immediate use. The adenylyl cyclase assay was carried out as described in Klotz et al., (1985), with minor modifications. Membranes were incubated for 30 min at 37°C in an incubation medium containing approximately 150,000 cpm of [a-^{32}P] ATP. To stop the reaction, the sample was precipitated on ice by adding 400 µl of 125 mM ZnAc and 500 µl of 144 mM Na_2CO_3, then it was vortexed and incubated for 10 min on ice. The supernatant was collected by centrifuging the sample in a benchtop centrifuge at maximal speed for 5 min at RT. 800 µl of the supernatant were loaded onto a column of alumina and eluted by washing twice with 2 ml 100 mM Tris, pH 7.4. The elution was collected in szintillation tubes and the cpm were counted in a β-counter. The potency of the diverse ligands in the adenylyl cyclase assay was calculated using the Hill equation. The dissociation constants (Ki) of the diverse antagonists were then calculated with the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

3.2.4 Transfection of MDA-MB-231 cells

For MKP-1 and/or MKP-2 knockdown, MDA-MB-231 cells were transfected with pools of MKP-2- and/or MKP-1-specific small interfering RNAs (siRNAs), respectively, or with a non target siRNA at 50 nM final concentration. Briefly, MDA-MB-231 cells were seeded at 30,000 cells/well in precoated six well plates in growth medium and transfected the next day when they were 60-70% confluent. The transfection was performed with Lipofectamine 2000 according to the manufacturer´s instructions in antibiotic and FCS free growth medium at 37°C in a 5% CO_2/95% atmosphere. Six hours after transfection of the cells, growth medium without antibiotic was added. The next day the medium was replaced with normal growth medium and the cells were incubated for an additional 24 hours. Forty-eight hours after transfection, cells were assayed for MKP-1 and MKP-2 protein expression. The transfected cells were stimulated with NECA and induction of MKP-1 and MKP-2 expression was determined by Western blot analysis.

3.2.5 Immunoblot analysis

Prior to an experiment, confluent MDA-MB-231 cells were washed with PBS, then trypsinized and seeded at 50,000 cells/well in precoated six well plates in medium containing 10% fetal calf serum. After 24 h, cells were treated with the indicated compound for the respective time periods. When inhibitors were used, they were added 30 min prior to the agonist. Agonist was added and incubated for the indicated times. After washing them with ice-cold PBS, cells were lyzed in electrophoresis sample buffer for 45 min on a shaker at RT, followed by sonification. In order to separate proteins of different length and estimate their size, they were analyzed using SDS-PAGE. The sodiumdodecylsulfate (SDS) denaturates the proteins and thus they can be separated in an electric field through the molecular sieve effect of the gel. The molecular weight of the protein equals the logarithm of the distance the protein traveled on the gel. Depending on the size of proteins of interest, gels with polyacrylamide content ranging from 10 - 12% were prepared as follows:

Polyacrylamide gels

12%	Lower (separating) gel, ml	Upper(stacking) gel, ml
37.5% Acrylamide	12.50	1.50
4 x Lower gel buffer	7.50	-
4 x Upper gel buffer	-	3.00
dH$_2$O	10.00	7.50
TEMED	0.015	0.012
10% APS	1.95	0.12
Total	30.00	12.00

The polymerisation takes 20-30 minutes. The gel was then run at 120 V. For Western blot or immunoblot analysis, proteins were transferred onto nitrocellulose membranes after separation by SDS-PAGE. Transfer was accomplished in a 'Wet'-blotting chamber applying 100 V for 1 h 15 min. Membranes with bound proteins served as platform for immune reactions. To reduce non-specific binding of the

antibody, the remaining binding sites of the positively charged nitrocellulose membranes were blocked with 5% dry milk powder for 90 min on a shaker at RT. Other blocking solutions, such as 3% BSA were also used if needed. Membranes were washed with several changes of wash buffer and probed with adequate primary antibody directed against the protein of interest, typically overnight at 4°C. The next day, the membrane was rinsed with washing buffer for 10 min, a total of six times, and the Fc chain of the bound primary antibody was detected after 1 h incubation with the appropriate secondary antibody coupled to horseradish peroxidase. After unbound antibody was removed by washing six times for 10 minutes and positive signals were detected by chemoluminescence using the ECL method, membranes were exposed to X-ray films and quantified by densitometry with ImageJ. In the case the same membrane had to be used in a second Western blot experiment, bound antibodies were removed by incubation with strip-buffer for 1 hour at 50°C. Then, membranes were thoroughly washed with TBS/0.1% Tween, blocked, and the whole process was repeated. An equal loading of protein was confirmed using anti-GAPDH or anti-β-tubulin antibody. For details see (Ruppert et al., 2013).

3.2.6 Measurement of intracellular Ca^{2+} in MDA-MB-231 cells

Concentrations of free intracellular Ca^{2+} were measured in cell suspension. Confluent MDA-MB-231 cells were washed briefly with PBS and trypsinized. The cells were diluted in the culture medium and pelleted by centrifugation. The pellets were washed and centrifugated two times with calcium loading buffer (CLB without glucose). The MDA-MB-231 cell pellets were loaded by incubating them for 45 min at 37°C in the dark in 10 ml CLB solution containing 2 μM Fura-2-AM, 0.04% Pluronic acid and washed twice with CLB, then placed in 6 ml CLB for another 30 min at room temperature to allow a complete de-esterification of Fura-2-AM. The cell suspension was stirred continuously throughout the experiment. The experiment was carried out in 2 ml of cell suspension and the fluorescence was measured with a Perkin-Elmer LS-50 B spectrofluorometer. Fura-2 fluorescence is emitted at 510 nm. The excitation wavelength alternated in intervals of 600 ms between 340 nm (bound Fura-2) and 380 nm (free Fura-2). The slit width was 10 nm, and the emission was measured at 510 nm. A 100-fold concentrated stock solution of different compounds was prepared in DMSO. The antagonists were added to the Fura-2-pre-loaded cells 15 min prior to

the agonists, which were added after a stable baseline was obtained. The intracellular free calcium concentration is given as the ratio of 340/380 nm. The maximal ratio (R_{max}) was determined for each experiment by successive addition of 0.02% Triton. The intracellular calcium in each experiment was calculated by the following formula:

$$[Ca^{2+}]_i = [(R - R_{min})/(R_{max} - R_{min})]$$

R_{max} = maximal ratio with 0.2% Triton-X100

R_{min} = baseline ratio

R = ratio after adding a ligand

3.3   Data analysis

All experiments were repeated at least 3 times. Statistical difference between two groups of data was tested by the paired Student's t test and the difference between three or more groups was assessed by a one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test. These analyses were performed using the statistical software package GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and a 0.05 significance level was assumed to indicate statistical significance. Results are expressed as treated/untreated ratio (mean ± SEM). For efficacy, data from each experiment were normalized to the maximal NECA effect and reported as percent of the maximal response.

4 Results

4.1 A$_{2B}$AR-mediated reduction of ERK1/2 phosphorylation in MDA-MB-231 cells

4.1.1 NECA induces a reduction of ERK1/2 phosphorylation

It was previously shown that MDA-MB-231 cells express a high level of A$_{2B}$ARs as the sole adenosine receptor subtype and exhibit a prominent basal ERK1/2 phosphorylation. In our study, the MDA-MB-231 cells were incubated either with increasing concentrations of NECA for 30 min or with 100 nM NECA for different time intervals. NECA, a nonselective A$_{2B}$AR agonist, reduced the phosphorylation of ERK1/2 phosphorylation (pERK1/2) in a concentration and time dependent manner (Fig. 10). Furthermore, a significant reduction in proliferation was observed upon NECA stimulation (Fig. 11).
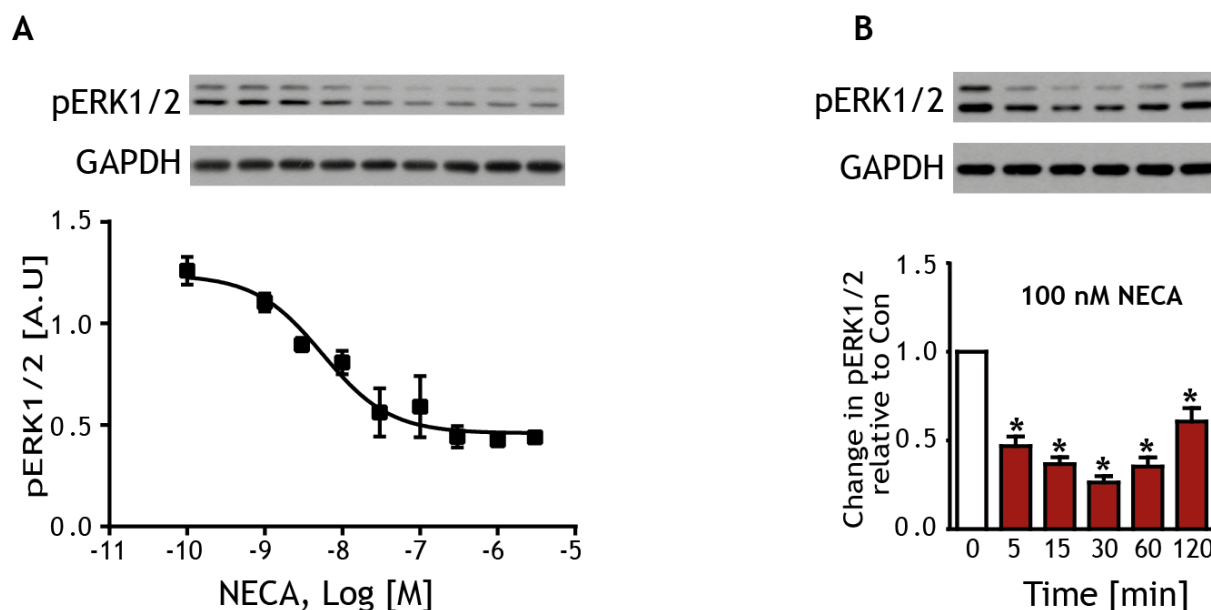
A

pERK1/2

GAPDH

B

pERK1/2

GAPDH

Figure 10: **Reduction of ERK1/2 phosphorylation by the adenosine receptor agonist NECA.** The MDA-MB-231 cells were stimulated for 30 min with indicated concentrations of NECA (A) or incubated with 100 nM NECA for the indicated times (B) prior to determination of ERK1/2 phosphorylation. (A) NECA reduced the ERK1/2 phosphorylation in a concentration-dependent manner (EC_{50} 5.85 nM, 95% confidence limit 2.91-11.8; n = 5). (B) The reduction was time-dependent with a maximum after 30 min. The Western blots in (A) and (B) show representative experiments. The columns in B show data from n = 7 independent experiments (* p < 0.001, significantly different from untreated cells).
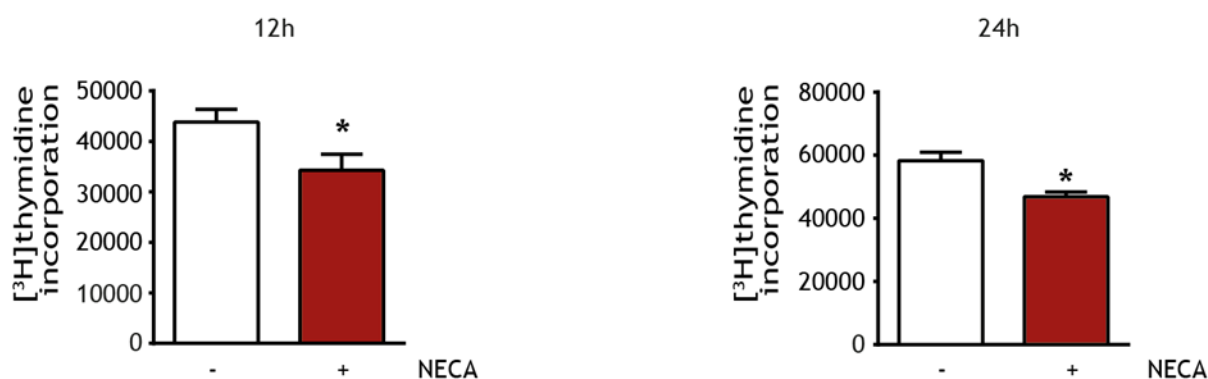
Figure 11: **NECA inhibits proliferation of MDA-MB-231 cells (experiment performed by Martina Fischer as described in (Schmid et al., 2015)).** MDA-MB-231 cell lines showed a significant decrease in cell proliferation when treated with NECA as judged by [^{3}H] thymidine incorporation. The proliferation is inhibited by about 80% after 12 and 24h of incubation with NECA. Data show mean values with SEM of n = 8 experiments, with each experiment performed in triplicates. The NECA values are significantly different from untreated cells with p = 0.0011 and 0.0004 for the 12 and 24h time points, respectively (paired Student's t-test).

4.1.2 Activation of the endogenous A$_{2B}$ARs mediates the reduction of ERK1/2 phosphorylation

Diverse AR agonists and antagonists of known specificity were tested for the reduction of ERK1/2 phosphorylation. None of the subtype selective agonists, including CCPA (A$_1$AR) and HEMADO (A$_3$AR), mimiced the NECA-mediated effect (Fig. 12A). The agonist CGS 21680, which activates all subtypes except A$_{2B}$AR at the concentration used (100 nM) caused no reduction of pERK1/2. A small reduction of pERK1/2 was observed at much higher concentration (10 μM) (Fig. 12B), likely due to A$_{2B}$AR stimulation. Moreover, the A$_{2A}$AR selective antagonist SCH 58261 and the A$_3$AR selective antagonist MRS 1220, did not antagonize the NECA-induced reduction of ERK1/2 phosphorylation (Fig. 12C-D). However, the nonselective antagonist EFA and the A$_1$/A$_{2B}$ antagonist DPCPX completely abolished the NECA-mediated reduction of ERK1/2 phosphorylation (Fig. 12E-F). Furthermore, DPCPX antagonized the NECA effect with an IC$_{50}$ of 203 nM consistent with its affinity to the A$_{2B}$AR (Fig. 56B). These results strongly point to the A$_{2B}$AR, the only AR subtype expressed in MDA-MB-231 cells, as the receptor responsible for the reduction of ERK1/2 phosphorylation.
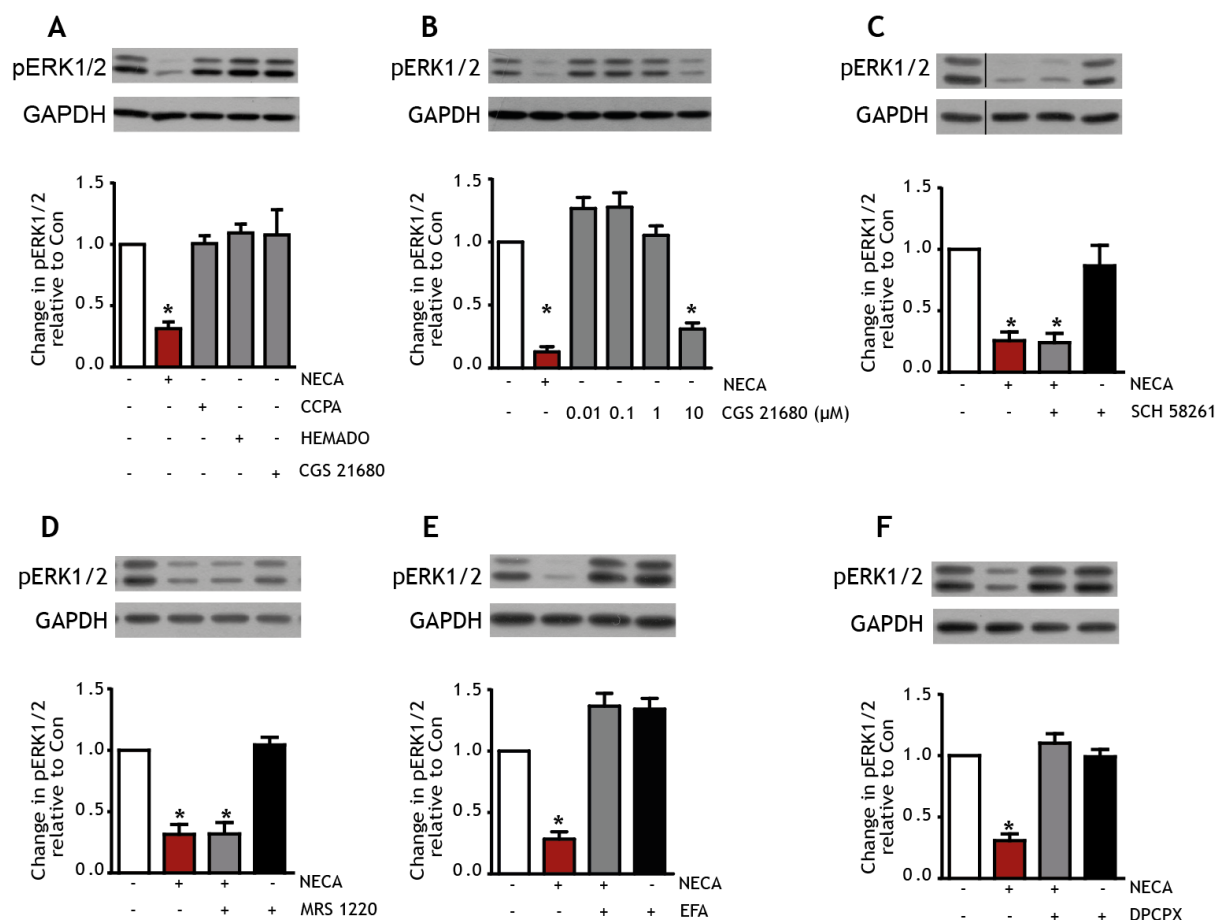
Figure 12: **NECA-stimulated ERK1/2 activation is mediated by A₂ʙARs.** (A) The nonselective AR agonist NECA induced a reduction of ERK1/2 phosphorylation. Activation of A₁AR with CCPA (100 nM), A₂ₐAR (also A₁AR and A₃AR to a certain degree) with CGS 21680 (100 nM), and A₃AR with HEMADO (100 nM) showed no effect. However, 10 μM CGS 21680 a concentration high enough to stimulate A₂ʙAR significantly reduced the ERK1/2 phosphorylation (B). The A₂ₐAR selective antagonist SCH 58261 (100 nM) (C) and the A₃AR selective antagonist MRS 1220 (100 nM) (D) did not reverse the NECA (100 nM) effect. In contrast, the nonselective antagonist EFA (10 μM) (E) and DPCPX at 10 μM, a concentration high enough to block A₂ʙAR (F) blocked the NECA (100 nM) effect. The Western blots show representative experiments, the columns represent mean values of n = 5 (A), 3 (B), 4 (C), 5 (D), 8 (E), and 6 (F) independent experiments, repectively (* p < 0.001, significantly different from untreated cells).

4.2 Adenylyl cyclase is necessary and sufficient for the reduction of ERK1/2 phosphorylation

The findings described above suggest that the reduction of ERK1/2 phosphorylation is an $A_{2B}AR$-mediated effect. Canonically, the $A_{2B}AR$ signaling pathway involves coupling to a G_s protein, stimulation of AC and cAMP production, followed by PKA activation (Linden et al., 1999). But other actions, including mobilization of intracellular calcium, have also been described (Panjehpour et al., 2005). To investigate the potential role of AC on ERK1/2 phosphorylation, we incubated the cells with 1 µM forskolin (FSK), an AC activator. FSK mediated a time-dependent reduction of ERK1/2 phosphorylation similar to that produced by NECA. To determine whether an increase in cAMP concentration mimics the $A_{2B}AR$-mediated reduction, we treated MDA-MB-231 cells with the cell permeable cAMP analoge cAMP-AM (100 µM), which releases cAMP after activation by esterases located inside the cell. In a second experiment, we applied the phosphodiesterase-4 inhibitor Ro 20-1724 for 30 min. We found that both compounds mimic the NECA-mediated reduction of ERK1/2 phosphorylation (Fig. 13). These results demonstrate that activation of AC, followed by an increase in cAMP, are necessary for the reduction of ERK1/2 phophorylation via $A_{2B}AR$.
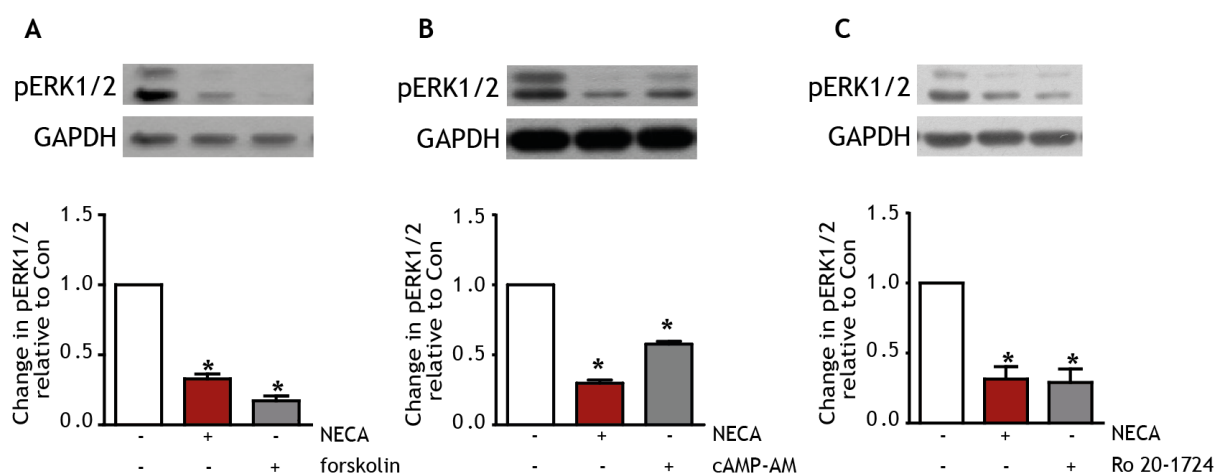
Figure 13: **Reduction of ERK1/2 phosphorylation by cAMP.** The effect of 100 nM NECA on ERK1/2 phosphorylation was mimicked by compounds increasing intracellular cAMP, like 1 µM forskolin (A), 100 µM cAMP-AM ("caged cAMP", B), or the PDE inhibitor Ro 20-1724 at 100 µM (C). The Western blots show representative experiments, the columns represent mean values from n = 6 (A), 4 (B), and 5 (C) independent experiments, respectively (* p < 0.001, significantly different from untreated cells).

4.3 Activity of cAMP-dependent protein kinase (PKA) is necessary for the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation

The primary downstream signal of cAMP is activation of PKA. We could show that PKA is also involved in the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation. Treatment with the widely used PKA inhibitor H89 (10 µM) completely abolished the reduction of ERK1/2 phosphorylation caused by NECA (Fig. 14A). However, KT 5720, a selective PKA inhibitor, failed to suppress the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation. Incubation with KT 5720 alone significantly reduced the basal phosphorylation of ERK1/2 (Fig. 14B).
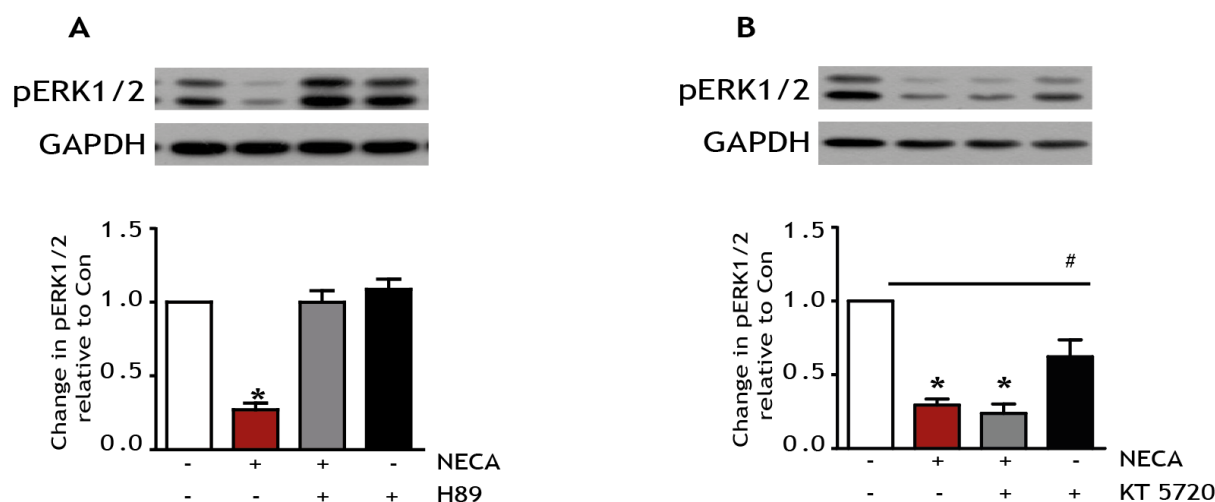
A

B

Figure 14: **Effect of PKA inhibitors on NECA-mediated reduction of ERK1/2 phosphorylation.** Preincubation (30 min) of MDA-MB-231 cells with 10 µM of the PKA inhibitor H89 (A) prevented the NECA effect on ERK1/2 phosphorylation, while inhibition of PKA with KT 5720 showed no effect on NECA-mediated reduction of pERK1/2. KT 5720 alone reduced the phosphorylation of ERK1/2 (B). The Western blots show representative experiments, the columns represent mean values from n = 7 (A), 5 (B) independent experiments, (* $p < 0.0001$, # $p < 0.05$, significantly different from untreated cells).

It is well known that PKA activation is involved in the phosphorylation of CREB at S333. In HEK293 cells, the activation of endogenous $A_{2B}AR$ was reported to induce a transient increase of ERK1/2 phosphorylation in a PKA dependent manner. To control the results of H89 and KT 5720 on PKA mediated ERK1/2 phosphorylation in MDA-MB-231 cells, we use the CREB phosphorylation at S133 in MDA-MB-231 cells, and the NECA-mediated ERK1/2 phosphorylation in HEK293 cells as control. We examined the NECA-mediated CREB-and ERK1/2 phosphorylation in MDA-MB-231 cells and HEK293 cells respectively in the presence and absence of H89 or KT 5720. As shown in Fig. 15, CREB phosphorylation was enhanced by NECA, and H89 almost abolished the increase without affecting the basal phosphorylation, while KT 5720 failed to reverse the phosphorylation (Fig. 15A-B). Incubation of HEK293 cells with 10 µM NECA for five minutes increased the ERK1/2 phosphorylation. Preincubation with H89 prevented the NECA-induced ERK1/2 phosphorylation, while inhibition of PKA with KT 5720 showed no effect (Fig. 15C-D). H89 abolished both the NECA-mediated CREB phosphorylation and ERK1/2 phosphorylation in MDA-MB-231 and HEK293 cells, respectively. The increase of pCREB and pERK1/2 was unaffected by KT 5720. The control experiments confirmed the KT 5720 results in MDA-MB-231 cells, suggesting

that KT 5720 is not an effective inhibitor of PKA. However, H89 may exert non-specific effect on other kinases.
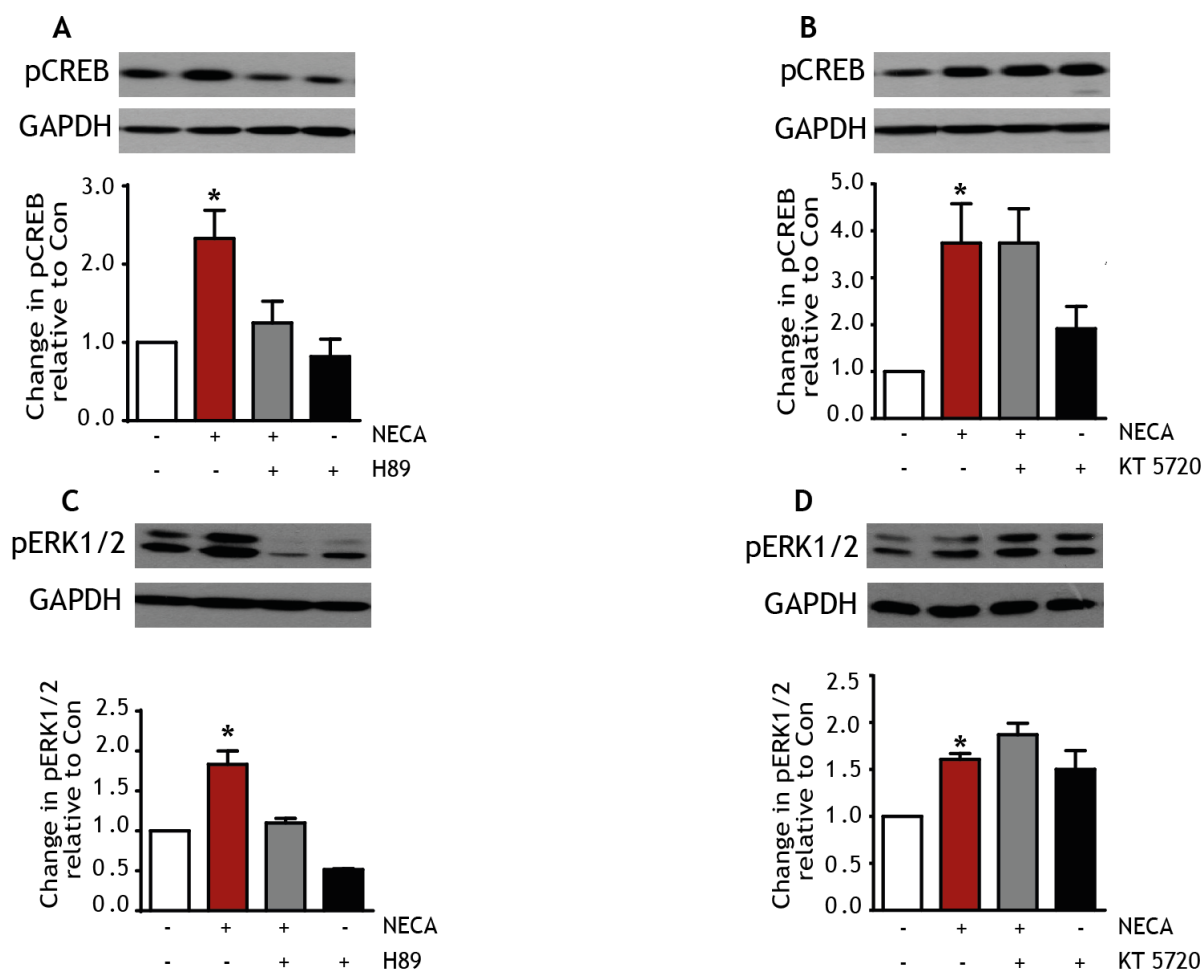

Figure 15: Control of PKA inhibitors H89 and KT 5720 responses. To confirm the results from Fig. 13, H89 and KT 5720 were tested on NECA-mediated CREB phosphorylation in MDA-MB-231 cells (A, B) or on NECA-mediated increase of ERK1/2 phosphorylation in HEK293 cells (C, D). 30 min preincubation of MDA-MB-231 cells with 10 µM of the PKA inhibitors H89 (A) prevented the NECA effect on CREB phosphorylation. While inhibition of PKA with KT 5720 (B) showed no effect. NECA-induced ERK1/2 phosphorylation in HEK293 cells was attenuated in the presence of H89 (C), KT 5720 failed to inhibit NECA-induced increase of pERK1/2 (D). The Western blots show representative experiments, the columns represent mean values from n = 3 independent experiments, (* p < 0.001, significantly different from untreated cells).

For many decades, PKA was as assumed to be the sole downstream target of cAMP, but it has been shown that cAMP also interacts with other targets, including a family of proteins called Epac (de Rooij et al., 1998; Kawasaki et al., 1998). Epac family members are guanine nucleotide exchange factors (GEFs) that catalyze the exchange of G-protein-bound GDP for GTP, rendering the G-protein active. To test for PKA-independent mechanisms downstream of cAMP, we stimulated the cells with the cAMP analog 8-CPT-2ME-cAMP, which specifically activates Epac but not PKA. Epac activation by 8-CPT-2Me-cAMP did not reduce the ERK1/2 phosphorylation (Fig. 16A). These results strongly point to a mechanism in which $A_{2B}AR$ activation enables PKA activation, which in turn participates in the reduction of ERK1/2 phosphorylation. Since H89 is not a selective PKA inhibitor, we had to rule out the possibility that H89 was blocking the reduction of pERK1/2 through PKC inhibition. In order to do that, MDA-MB-231 cells were pretreated with the PKC inhibitor GF-109203X, followed by NECA treatment for 30 min. In addition, cells were treated with PMA, a PKC activator for 30 min. Neither PKC inhibition abolished the reduction of ERK1/2 phosphorylation, nor did PKC activation provoke the reduction of ERK1/2 phosphorylation. PKC does not seem to be involved in the reduction of ERK1/2 phosphorylation (Fig. 16B-C).
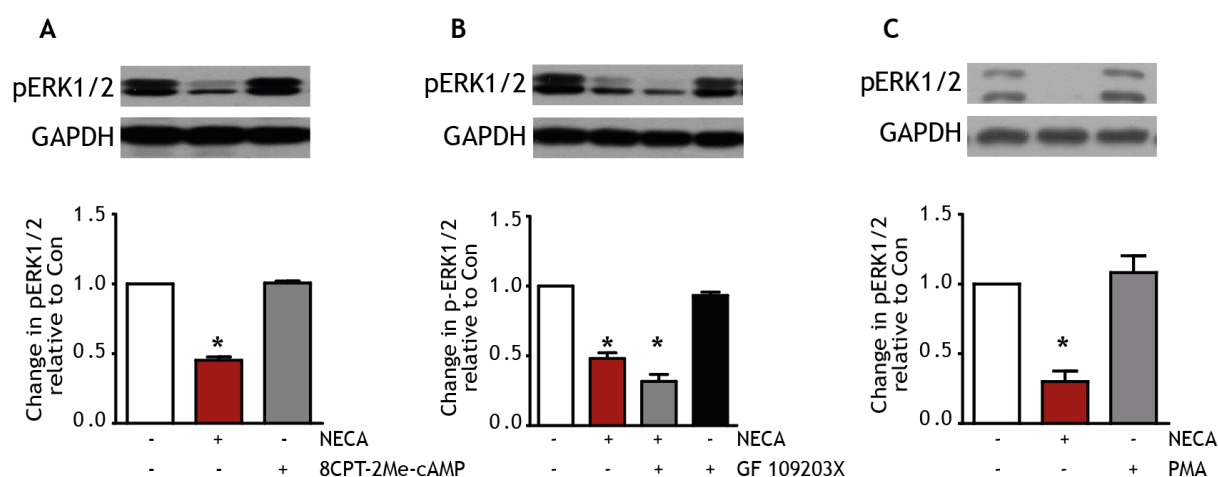
Figure 16: (A) The effect of 100 nM NECA on ERK1/2 phosphorylation was not mimicked by the selective membrane permeant EPAC agonist 8CPT-2Me-cAMP. Preincubation (30 min) of MDA-MB-231 cells with 10 µM of the PKC inhibitor GF 109203X did not prevent the NECA effect on ERK1/2 phosphorylation (B). PKC activation by 1 µM PMA for 30 min showed no effect (C). Representative immunoblots of pERK1/2 (top) and GAPDH (bottom) are shown, the columns represent mean values from n = 3 (A), 3 (B), 5 (C), independent experiments, respectively (* p < 0.001, significantly different from untreated cells).

4.4 Phospholipase C (PLC) activation is required for the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation

Even though the $A_{2B}AR$ was characterized as a low affinity receptor, NECA reduced the ERK1/2 phosphorylation with a high potency (EC_{50} = 5.85 nM) (Fig.10A) in MDA-MB-231 cells. This is in clear contrast to the EC_{50} of 427 nM for cAMP (Fig. 48) production in the same cells. This suggests the presence of some amplifying pathway(s) downstream of $A_{2B}AR$, in addition to the AC-cAMP-PKA pathway, making the NECA mediated reduction of ERK1/2 phosphorylation more potent. In addition to AC activation, the $A_{2B}AR$ has been linked to mobilization of calcium through the activation of PLC. This prompted us to investigate whether PLC might be involved in the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation. For this purpose, MDA-MB-231 cells were treated with a PLC inhibitor. As shown in Fig. 17A, inhibition of PLC with U73122 blocked $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation, whereas U73343, the inactive analog, did not abrogate the reduction of ERK1/2 phosphorylation (Fig. 17B). These results strongly suggest a mechanism in which $A_{2B}AR$ activation leads to PLC activation, which in turn contributes to the reduction of ERK1/2 phosphorylation.
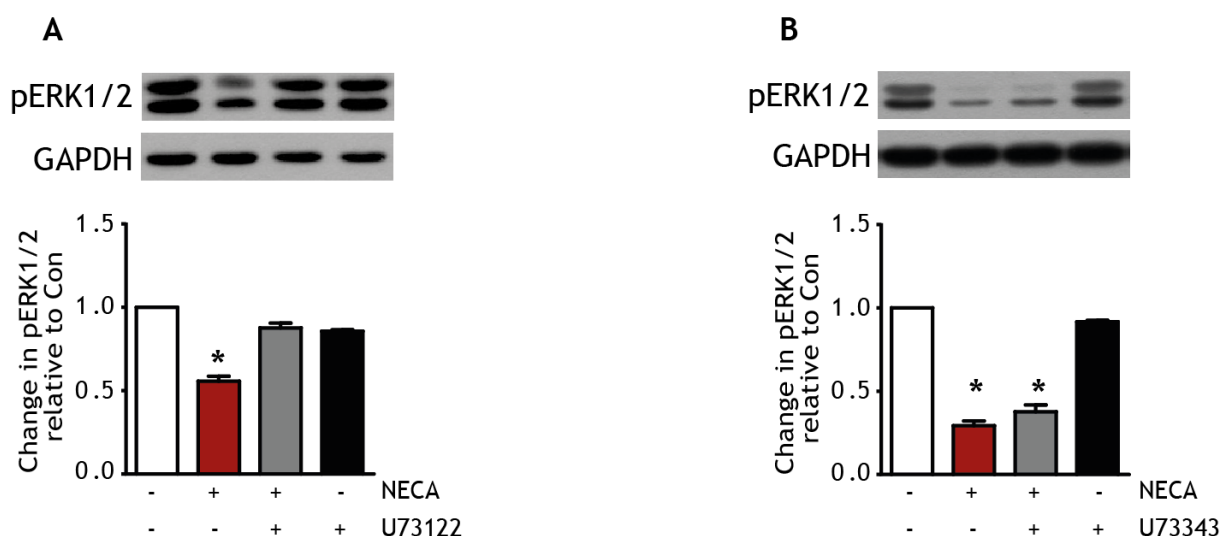
Figure 17: **NECA-mediated reduction of ERK1/2 phosphorylation is sensitive to PLC inhibitor.** The MDA-MB-231 cells were preincubated for 30 min with 10 µM of PLC inhibitor U73122 (A) or the inactive analog U73343 (B) prior to stimulation with 100 nM NECA. The Western blot shows representative experiments, the columns represent mean values from $n = 7$ (A), 3 (B) independent experiments, respectively (* $p < 0.001$, significantly different from untreated cells).

4.5 Intracellular calcium mobilization is involved in the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation

Once activated, PLC cleaves PIP_2 into DAG and IP_3. IP_3 binds to a specific IP_3 receptor (calcium channel) in the endoplasmic reticulum and causes an increase of cytosolic calcium concentration. To test for calcium-dependent mechanisms downstream of PLC activation, the cells were treated with BAPTA-AM, a calcium chelator. The cell permeable Ca^{2+}-chelating agent (BAPTA-AM) abolished the $A_{2B}AR$ effect (Fig. 18A). Furthermore, incubation of MDA-MB-231 cells with UTP led to the reduction of ERK1/2 phosphorylation (Fig. 18B). UTP is an agonist for $P2Y_{2/4}$ receptors, which couples to Gq and triggers a calcium response (Fig. 18C). These data indicate that calcium accumulation alone is sufficient for the NECA-stimulated reduction of ERK1/2 phosphorylation. Therefore, $A_{2B}AR$ mediated reduction of ERK1/2 phosphorylation appears to be enabled by PKA as well as by calcium release.
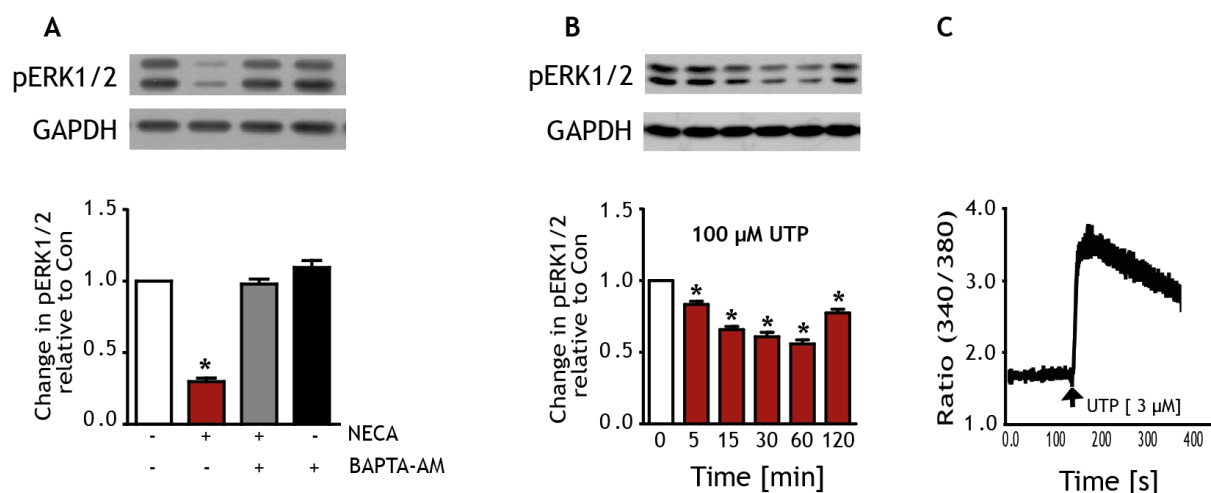
Figure 18: **Role of Ca^{2+} in the reduction of ERK1/2 phosphorylation.** Incubation with 30 µM of the cell-penetrating compound BAPTA-AM results in intracellular release of the Ca^{2+}-chelator BAPTA, which blocked the NECA-induced reduction of ERK1/2 phosphorylation (A). Consequently, an intracellular increase of Ca^{2+} triggered by 100 µM UTP (C) resulted in a reduction of ERK1/2 phosphorylation (B). The Western blots and the Ca^{2+} trace in C show representative experiments, the columns represent mean values of n = 7 (A) and 8 (C) independent experiments, respectively (* p < 0.001, significantly different from untreated cells).

4.6 The reduction of ERK1/2 phosphorylation requires *de novo* protein synthesis

To investigate whether A$_{2B}$AR-mediated reduction of ERK1/2 phosphorylation may require *de novo* protein synthesis, the MDA-MB-231 cells were pretreated for 30 min with cycloheximide (CHX), an inhibitor of protein translation, followed by 30 min of treatment with NECA. Pretreatment of MDA-MB-231 cells with CHX completely blocked the NECA-induced reduction of pERK1/2 (Fig. 19). These results suggest that *de novo* protein synthesis plays a critical role in the reduction of ERK1/2 phosphorylation.
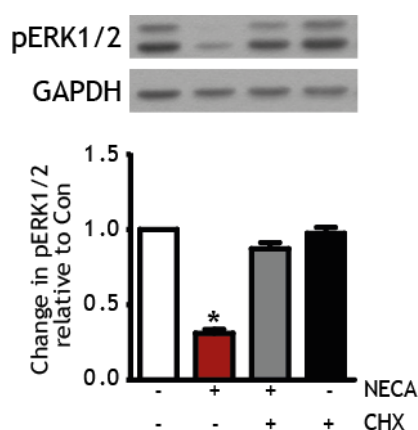
Figure 19: Effect of cycloheximide on ERK1/2 phosphorylation. The reduction of ERK1/2 phosphorylation by NECA was blocked by 10 µg/ml of cycloheximide (CHX) suggesting that protein synthesis is mandatory for the NECA effect. The Western blot shows a representative experiment, the columns represent mean values from n = 7 independent experiments (* $p < 0.001$, significantly different from untreated cells).

4.6.1 MKP-1 expression in MDA-MB-231 cells

Phosphorylation of threonine and tyrosine residues is required for MAPK activation (Chang and Karin, 2001). Since MAPKs are regulated by reversible phosphorylation, inhibition of MAPK phosphorylation can be controlled either through dephosphorylation (activation of a phospahatase), or through inhibition of upstream activators (upstream kinases). A computational analysis of the MAPK system suggests that the MKPs dictate the extent of MAPK phosphorylation (Bhalla et al., 2002). The MKPs are a family of eleven dual-specificity Thr/Tyr MAPK phosphatase, which act as negative feedback regulators of MAPK signaling (Dickinson and Keyse, 2006; Keyse, 2000). Among them, MKP-1 and MKP-2 are encoded by highly inducible genes, which are rapidly up-regulated in response to both mitogenic and/or stress stimuli at the transcriptional level. Both phosphatases are located in the nucleus and have been shown to dephosphorylate MAPKs. MKP-1 and MKP-2 have distinct patterns of substrate specificity. MKP-1 and MKP-2 recognize ERK2, JNK2, and p38, ERK2 and JNK2, respectively (Chu et al., 1996). Due to these findings, we examined the expression of the MAPK phosphatase MKP-1 and MKP-2 following $A_{2B}AR$ activation and evaluated the effects of MKP-1 expression on ERK1/2 phosphorylation. MDA-MB-231 cells were treated with 100 nM NECA for different periods of time and induction of the MKP-1 protein was determined by Western blot analysis. We observed an increase in MKP-1 expression as a result of NECA treatment. Time-course experiments

revealed that MKP-1 protein, which was barely present under basal condition increased after NECA treatment, reaching a maximum concentration after approximately 30 min, before returning to basal values within 2 hours (Fig. 20A). Also, stimulation with the AC activator forskolin for 30 min induced an increased MKP-1 protein expression (Fig. 20B). The protein synthesis inhibitor CHX blocked these responses when added 30 min before the stimulus (Fig. 20C). Thus, the inhibiton of MPK-1 expression by CHX seems to account for the inhibition of the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation by CHX. MKP-1 seemed to be the CHX sensitive protein needed for the reduction of pERK1/2. Taken together, our results suggest that MKP-1 expression might play a critical role in the reduction of ERK1/2 phosphorylation in MDA-MB-231 cells, and that the MKP-1 expression is dependent on AC activity and cAMP.
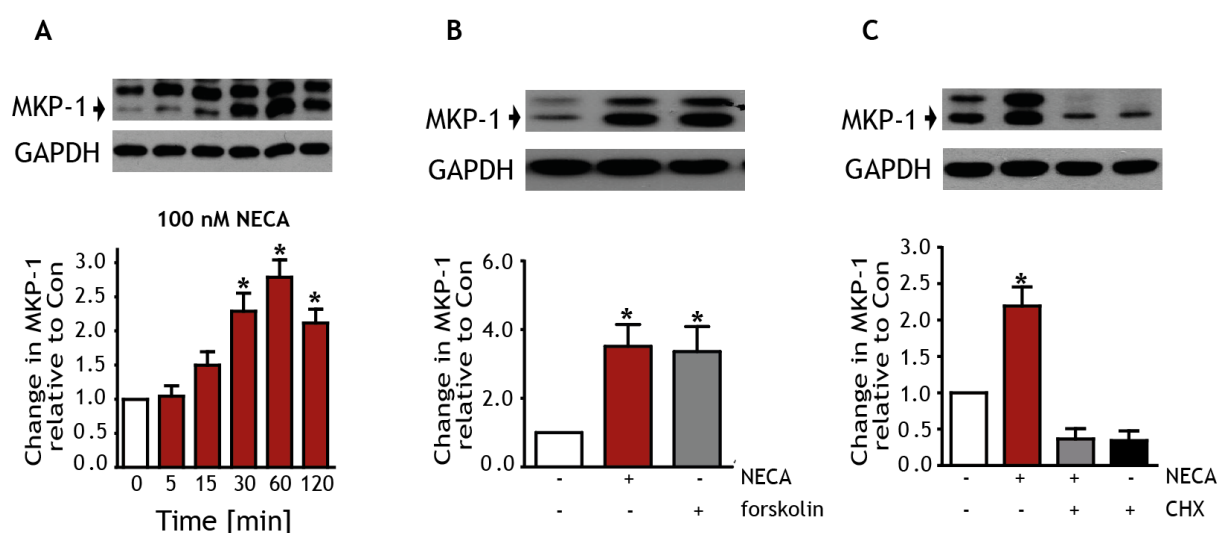

Figure 20:   MKP-1 expression. Stimulation of $A_{2B}AR$ in MDA-MB-231 cells with NECA causes an increase in MKP-1 expression (A), treatment with 1 µM forskolin shows the same effect (B). The increase caused by NECA is blocked by cycloheximide (C). The Western blots show representative experiments, the columns represent mean values from n = 4 (A), 7 (B), 6 (C) independent experiments, respectively (* $p < 0.001$, significantly different from untreated cells).

Since our previous results revealed that AC- and calcium-pathways are involved in the reduction of ERK1/2 phosphorylation, we then determined whether an increase in the intracellular concentration of Ca^{2+} could also provoke MKP-1 gene expression. MDA-MB-231 cells were treated with UTP in the presence or absence of forskolin and

the level of MKP-1 expression was monitored. As shown in Fig. 21A, addition of UTP is sufficient to stimulate an increase in the expression of MKP-1 protein. Futhermore, UTP increased the forskolin-mediated expression of the MKP-1 protein, while the calcium chelator BAPTA-AM blocked MKP-1 expression (Fig. 21B). These results indicate that Ca^{2+} is required for MKP-1 gene expression.
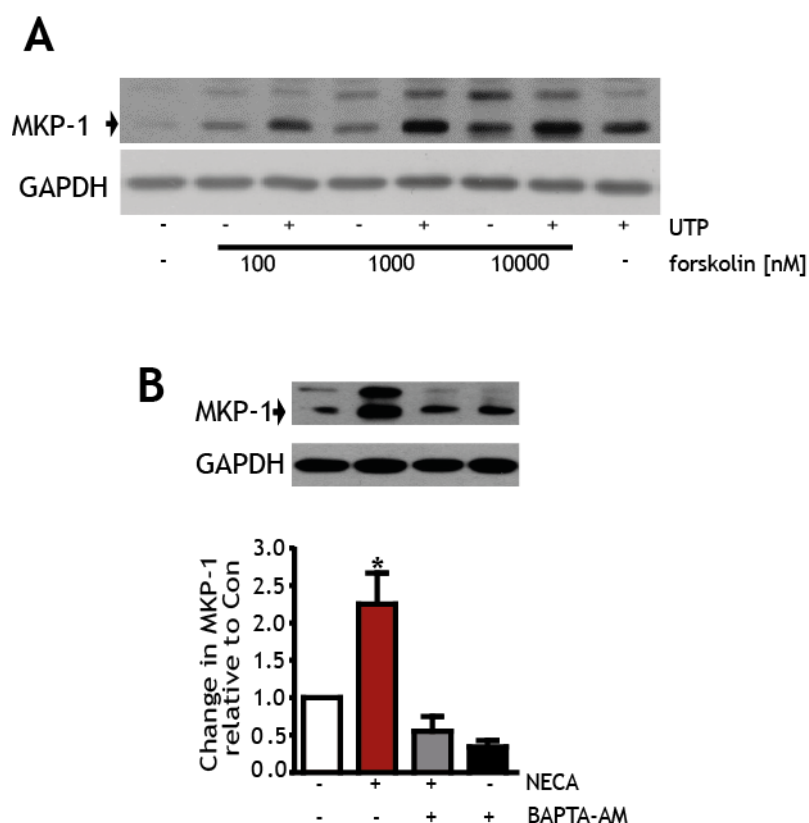

Figure 21: **Ca^{2+} effect on MKP-1 expression**. Incubation of the MDA-MB-231 cells with 100 µM UTP induces MKP-1 expression and consequently increases the FSK-mediated MKP-1 expression (A). Addition of the Ca^{2+}-chelator BAPTA-AM (30 µM) blocked the NECA-induced increase of MKP-1 expression (B). The Western blot shows a representative experiment, the columns represent mean values from n = 4 independent experiments (* p < 0.001, significantly different from untreated cells).

The preceding outcomes, together with the ability of Ca^{2+}-chelating agents to block MKP-1 expression and abrogate the reduction of ERK1/2 phosphorylation, support a role of intracellular calcium mobilization in MKP-1 expression, and for MKP-1 to act as an ERK1/2 phosphatase in MDA-MB-231 cells. Furthermore, sanguinarine, a MKP-1 inhibitor, abolished the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation and

provided biochemical evidence that MKP-1 negatively regulates the ERK1/2 phosphorylation in MDA-MB-231 cells (Fig. 22A). A discrepancy in the time course of the reduction of ERK1/2 phosphorylation and the MKP-1 expression was observed. While the expression of MKP-1 increased significantly within 30 min, the reduction of ERK1/2 phosphorylation started already after 5 min. The time-discrepancy suggests that another phosphatase, or a kinase, could be involved in the reduction of ERK1/2 phosphorylation. In line with this, the reduction of the ERK1/2 phosphorylation in the NECA-treated cells was sensitive to the tyrosin phosphatase inhibitor sodium orthovanadate (Fig. 22B). This proves that the activity of phosphatases like DUSP and PTP are required for the $A_{2B}AR$-dependent reduction of ERK1/2 phosphorylation.
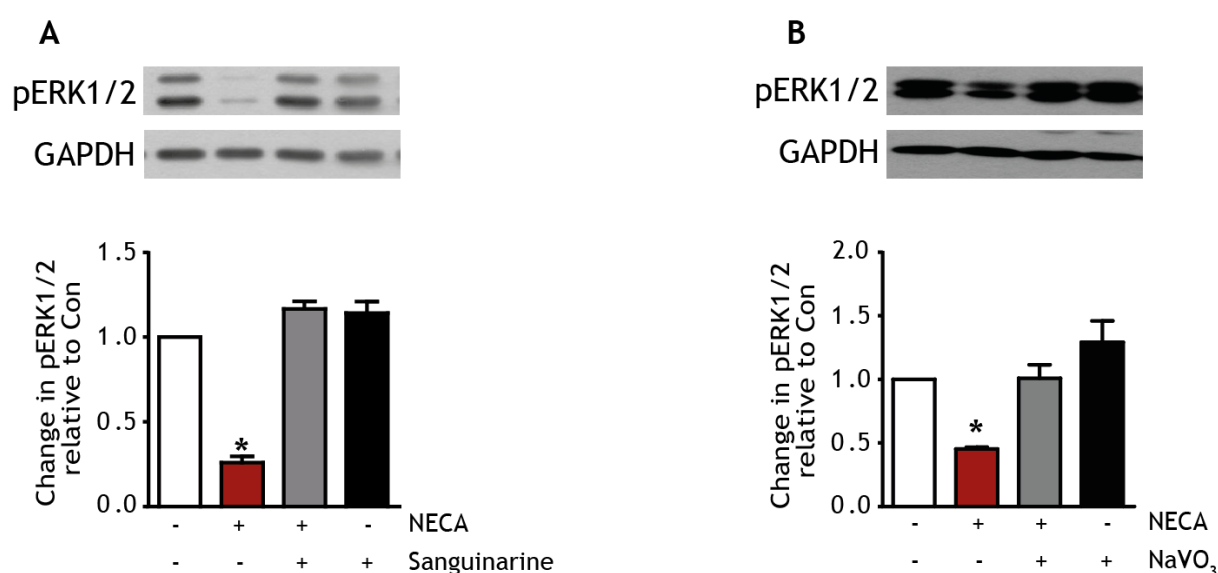
Figure 22: **Effect of phosphatase inhibitors on ERK1/2 phosphorylation.** The inhibition of ERK1/2 phosphorylation by NECA was blocked by 10 µM sanguinarine (A) and by 100 µM $NaVO_3$ (B) both phosphatases inhibitors. Suggesting that activities of phosphatases are required for the NECA-mediated reduction of pERK1/2. The Western blots show representative experiments, the columns represent mean values from n = 7(A), 3(B) independent experiments, respectively (* $p < 0.001$, significantly different from untreated cells).

The Western blot analysis with anti-MKP-1 antibody showed the presence of two single bands with apparent molecular masses of 40 and 42 kDa. To confirm the nature of the different bands and to further establish that the expression of MKP-1 can

reduce ERK1/2 phosphorylation, we measured the effects of MKP-1 knockdown with siRNAs on ERK1/2 phosphorylation upon $A_{2B}AR$ stimulation. MDA-MB-231 cells were transiently transfected with either a non target siRNA or with MKP-1- or MKP-2 siRNA constructs. The siRNA-assay confirmed the identity of the two bands. MKP-2 siRNAs reduced the upper band compared to the non-silencing controls, while MKP-1 siRNA reduced the lower band, suggesting that the upper band detected by the MKP-1 antibody is indeed MKP-2 (Fig. 23).
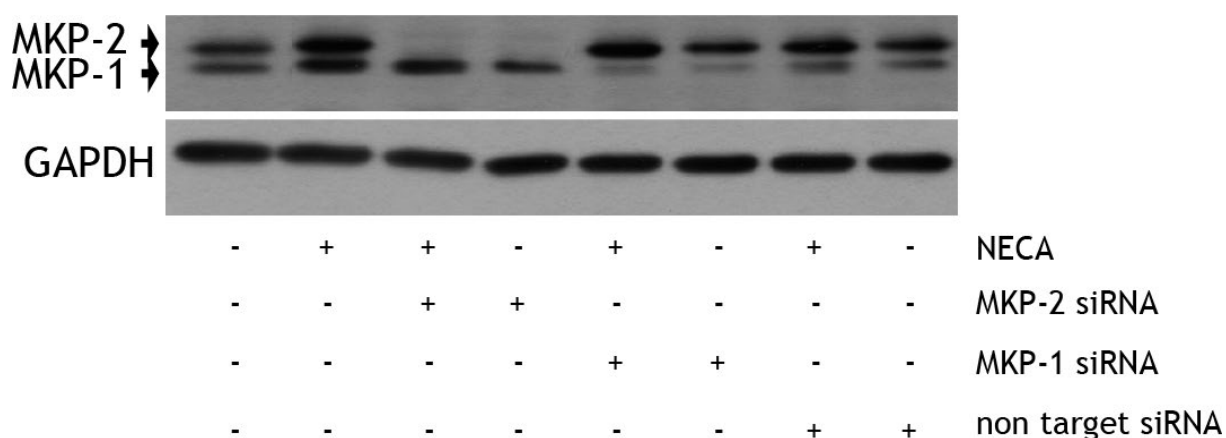

Figure 23: siRNA-based MKP-1 and MKP-2 silencing. MKPs expression after NECA treatment in MDA-MB-231 cells transfected with 50 nM siRNA targeting either MKP-1 or MKP-2 or 50 nM with non target siRNA for 48 hours then stimulated with or without 100 nM NECA for 30 min and then subjected to Western blot analysis. Representative immunoblots showing the protein levels of MKP-1 (top panel, lower band), MKP-2 (top panel, upper band) and glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) (bottom panel) is included to demonstrate loading consistency.

4.6.2 MKP-2 expression in MDA-MB-231 cells

Since the siRNA assay revealed that the upper band observed with the MKP-1 antibody is MKP-2, we analyzed our results again by quantifying the upper band. We observed a time dependent increase in MKP-2 protein levels after the activation of $A_{2B}AR$ (Fig. 24B). Furthermore, a latent increase of MKP-2 protein level was observed by using the MKP-2 antibody (Fig. 24A), confirming the MKP-1 antibody results. As opposed to MKP-1, MKP-2 was significantly increased after 15 min. The agents that increased or blocked MKP-1 expression or activity displayed the same effect on MKP-2 protein (blot 20B, upper lane; Fig. 24C-D).
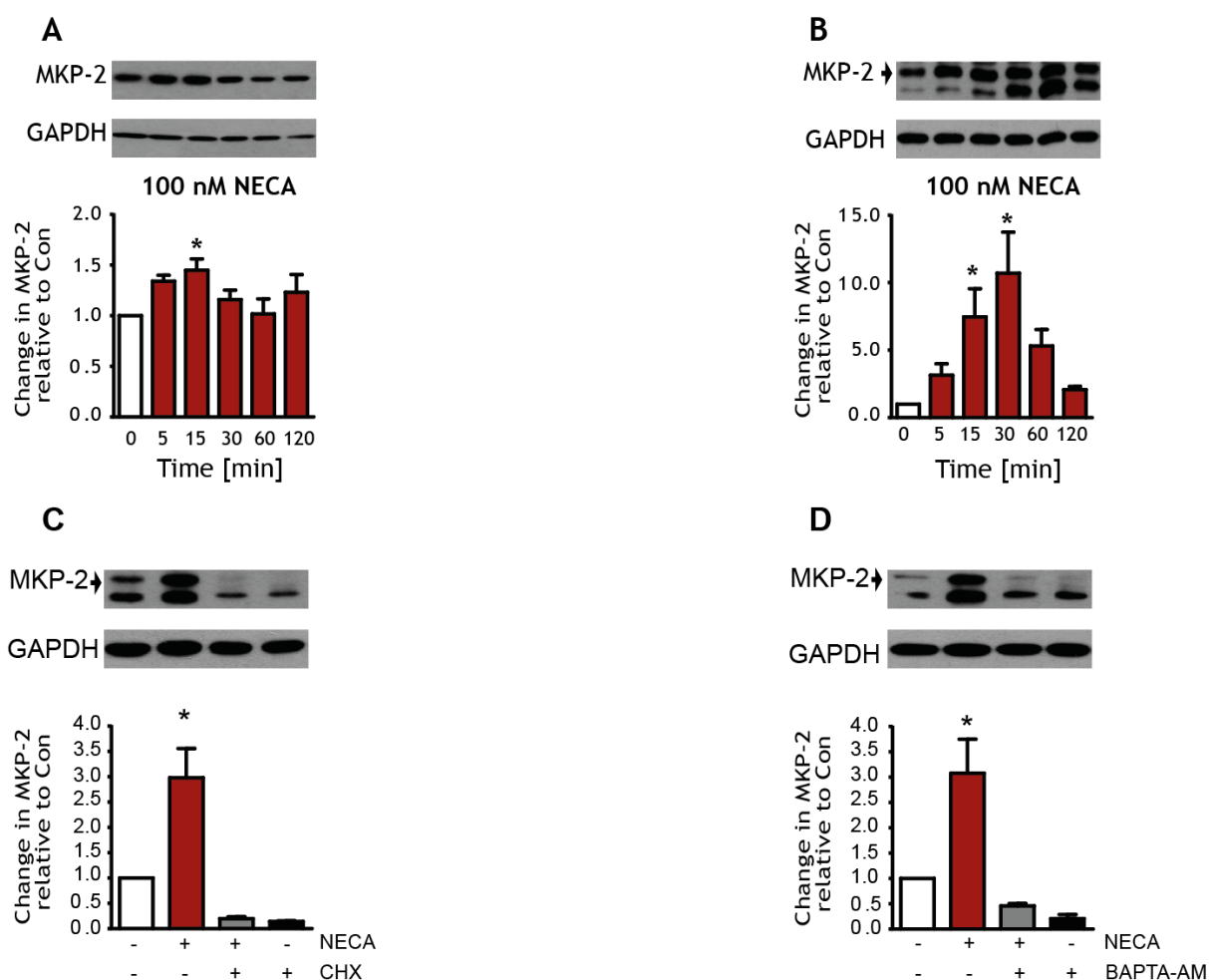
Figure 24: **MKP-2 expression**. Stimulation of $A_{2B}AR$ in MDA-MB-231 cells with NECA caused an increase in MKP-2 expression (A-B). The increase caused by NECA is blocked by 30 min preincubation with 10 µg/ml CHX (C), or with 30 µM of the Ca^{2+}-chelator BAPTA-AM (D). The Western blots show representative experiments, the columns represent mean values from n = 5 (A), 4 (B), 6 (C) and 4 (D) independent experiments, respectively (* $p < 0.05$, (A), * $p < 0.01$(B-D), significantly different from untreated cells).

NECA induced an increase in MKP-1 and MKP-2 expression. These effects were reversed by transfection with the specific MKP siRNA. However, as shown in Fig. 25, neither a separate nor a simultaneous knockdown of MKP-1 and MKP-2 proteins reversed the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation.

Figure 25: **siRNA-based MKP-1 and MKP-2 silencing and ERK1/2 phosphorylation.** MDA-MB-231 cells were transfected with 50 nM siRNA targeting either MKP-1, MKP-2, or MKP-1 and MKP-2 together with 50 nM non target siRNA as control for 48 hours. 100 nM 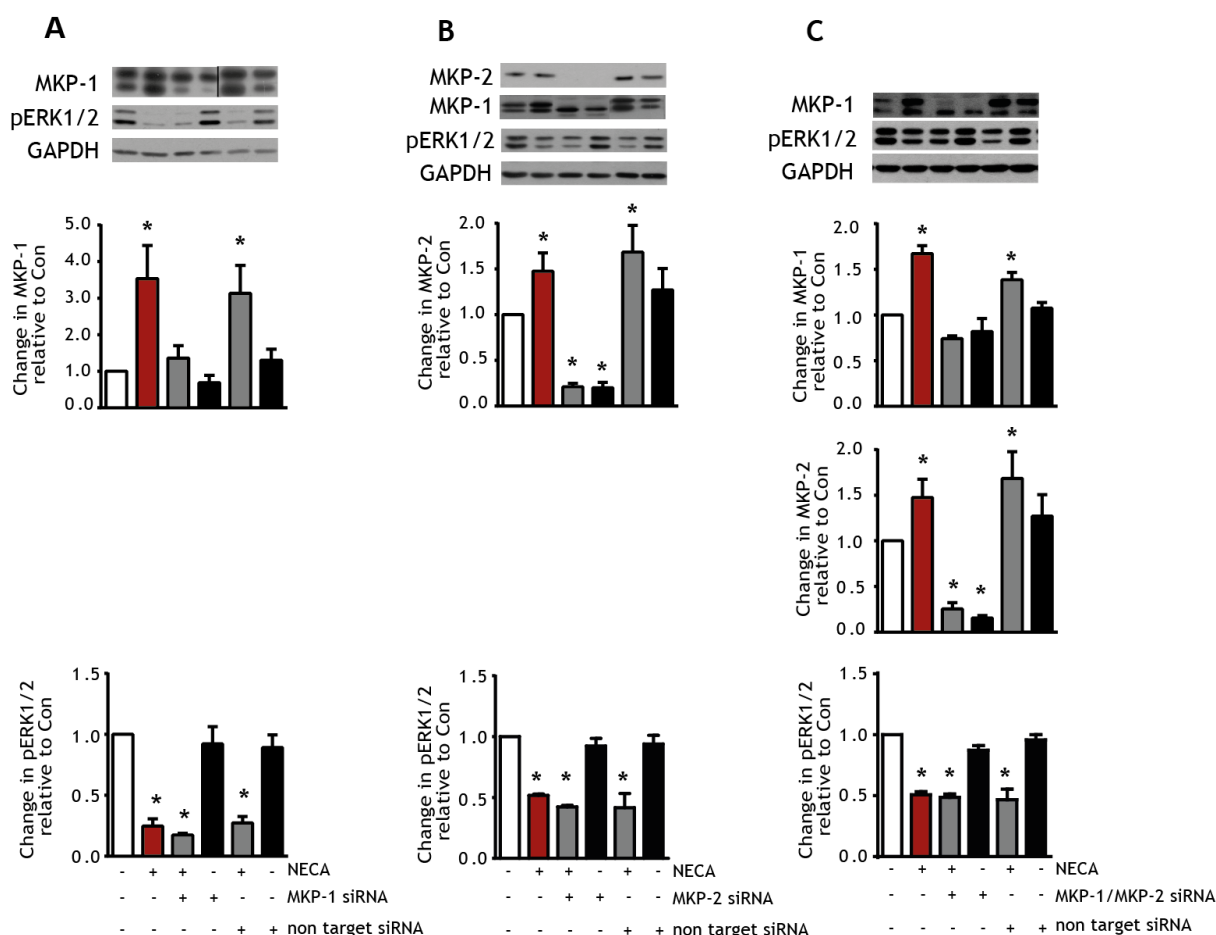NECA was added for the last 30 min (upper and midle panel A-C). Expression of MKP-1, MKP-2 and phosphorylation of ERK1/2 was examined concomitantly in the same samples by Western blot analysis with specific antibodies. A significantly reduction was noticed in NECA induced MKP expression in cells transfected with MKP-1 and MKP-2 siRNA, but not in cells transfected with non target siRNA (A-C, top and middle panel). In contrast MKP knockdown was not sufficient for inhibition of NECA-mediated pERK1/2 reduction (A-C, bottom panel). Representative immunoblots showing the protein levels of MKP-1, MKP-2, pERK1/2 and GAPDH are included to demonstrate loading consistency. The bar graphs show the quantification of n = 3 independent experiments, respectively (* $p < 0.01$ significantly different from untreated cells).

This might be attributable to incomplete silencing of the MKP-1 gene. While the siRNA-assay showed a total knockdown of MKP-2 proteins, a completely knockdown of the MKP-1 protein could not be achieved. Furthermore, increased MKP-2 protein levels were detected following NECA treatment in MKP-1 knockdown cells and vice

versa. Consequently, MKP knockdown was not sufficient for inhibition of NECA-mediated pERK1/2 reduction (Fig. 26). MKP-1 and MKP-2 may partially substitute for each others activity. The increase of MKP-1 and MKP-2 protein expression suggests a redundant mechanism that may ensure the reduction of ERK1/2 phosphorylation after $A_{2B}AR$ stimulation.
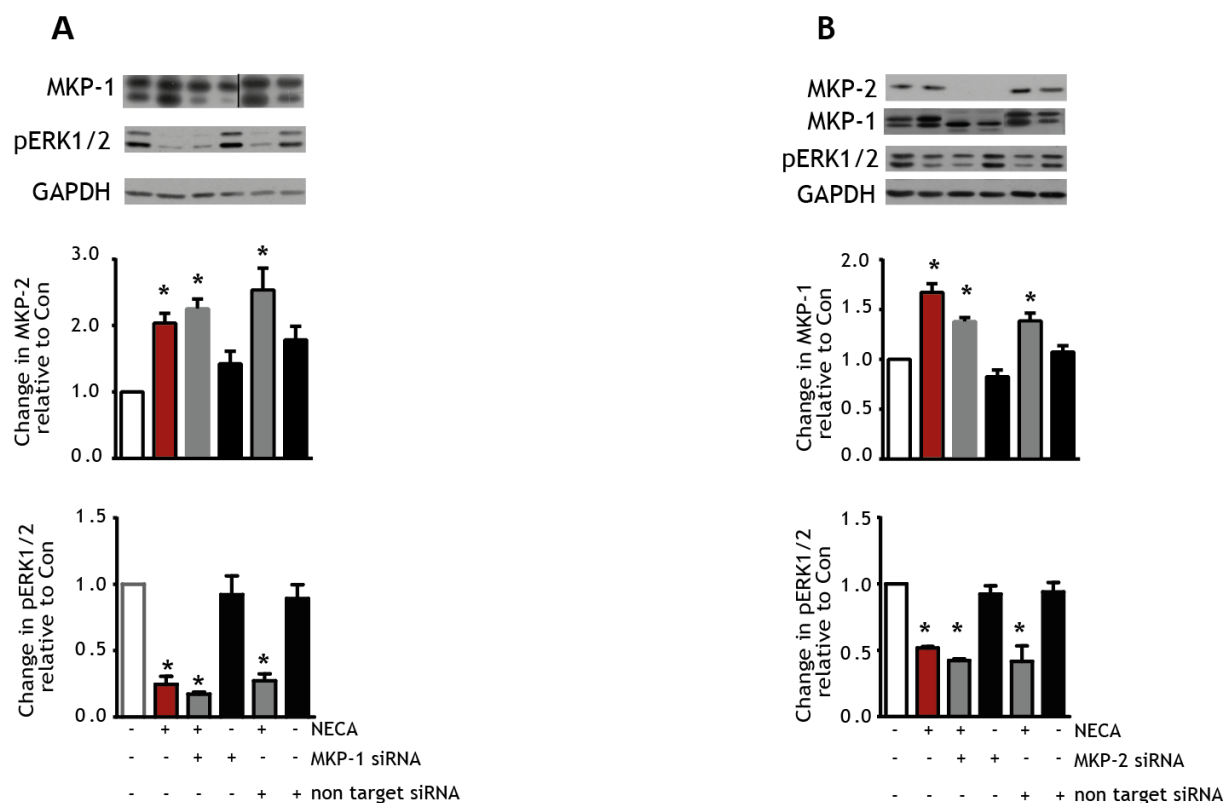
Figure 26: **siRNA-based MKP-1 and MKP-2 silencing and ERK1/2 phosphorylation.** MDA-MB-231 cells were transfected with 50 nM siRNA targeting either MKP-1 or MKP-2, or with 50 nM non target siRNA as control for 48 hours. NECA (100 nM) was added for the last 30 min. Expression of MKP-1 in MKP-2 siRNA treated cells, MKP-2 in MKP-1 siRNA treated and phosphorylation of ERK1/2 was examined concomitantly in the same samples by Western blot analysis with specific antibodies. A significant expression of MKP-2 and MKP-1 was noticed respectively, in MKP-1 siRNA and MKP-2 siRNA cells (A-B, top panel). Consequently, MKP knockdown was not sufficient for inhibition of NECA-mediated pERK1/2 reduction (A-B, bottom panel). Representative immunoblots showing the protein levels of MKP-1, MKP-2, pERK1/2 and GAPDH as a loading control are included. The bar graph shows the quantification of at least n = 3 independent experiments (* p < 0.01 significantly different from untreated cells).

4.6.3   Post-translational regulation of MKP-1 and MKP-2 in MDA-MB-231 cells

Phosphorylation of two serines in the C-terminus of both MKP-1 and MKP-2 has been shown to stabilize these proteins (Brondello et al., 1999; Crowell et al., 2014). We considered the possibility of MKPs to be controlled through a post-translational mechanism. We asked the question whether MKP-1 and MKP-2 stability was affected by $A_{2B}AR$ stimulation in MDA-MB-231 cells. When monoclonal anti-phospho-DUSP1/MKP1 (Ser359) antibodies were used, we observed two distincts bands, which were decreased upon NECA stimulation. These results suggest that in the MDA-MB-231 cells, MKP-1 and MKP-2 were phosphorylated under basal conditions and NECA treatment results in dephosphorylation of both proteins (Fig. 27). This may represent an inhibitory feedback mechanism to restrict the duration of MKP activity.
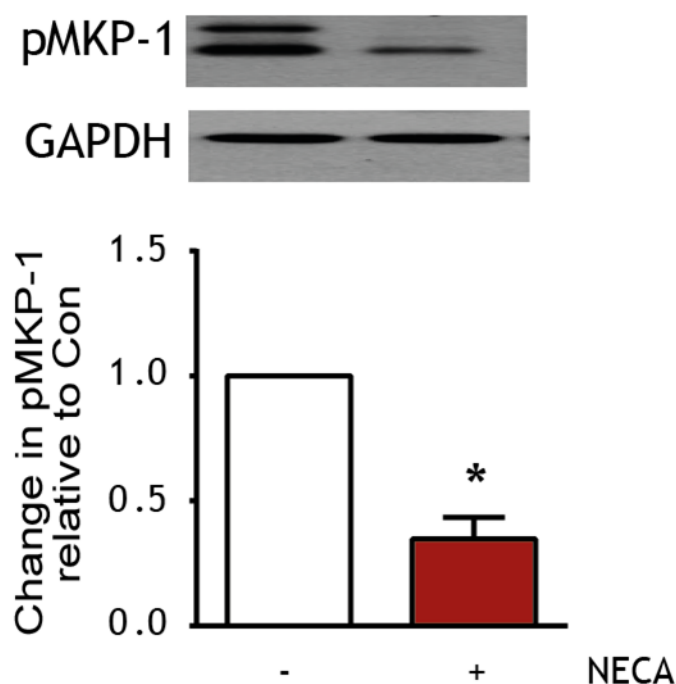

Figure 27:   **Reduction of MKP-1 phosphorylation by NECA.** MDA-MB-231 cells were treated with 100 nM NECA for 30 min. The Western blots show a representative experiment, the columns represent mean values from n = 6 independent experiments (* $p < 0.0001$ significantly different from untreated cells).

4.7 Synergism of cAMP and calcium signaling pathways leading to reduction of ERK1/2 phosphorylation

Increasing cAMP levels, e.g. by treatment with the AC activator forskolin, cause a reduction of ERK1/2 phosphorylation (Fig. 13A). Similarly, increasing intracellular calcium levels induced by 100 µM UTP also provoked a transient reduction of ERK1/2 phosphorylation (Fig. 18B). As opposed to UTP, forskolin did not trigger calcium mobilization in MDA-MB-231 cells. Similarly, UTP (100 µM) did not affect the EC_{50} of cAMP production in response to forskolin (Fig. 28A), nor did increasing UTP concentrations reverse the maximal forskolin-mediated cAMP production (Fig. 28B).

A

B
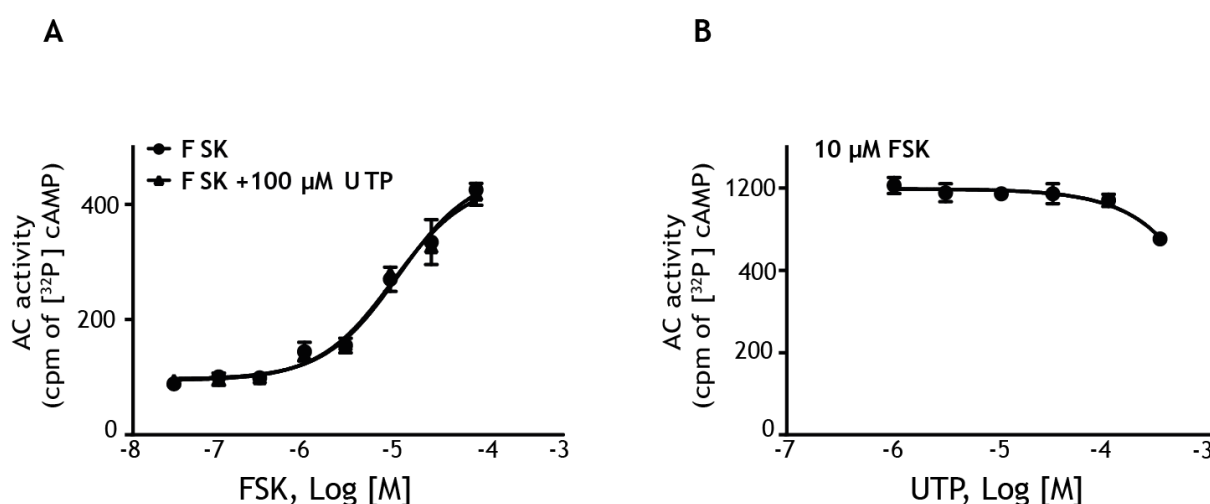
Figure.28: **Concentration-response curves** for agonist-stimulated cAMP production in MDA-MB-231 cells giving an EC_{50} of 12.5 µM for FSK alone, and of 10.9 µM for FSK in the presence of 100 µM UTP (A). For UTP an EC_{50} could not be determined (B). The data are means ± S.E.M of a typical experiment that was performed in duplicate three times.

To examine whether cAMP and calcium pathways act synergistically, we treated the MDA-MB-231 cells simultaneous with 30 nM of forskolin, a concentration which has no effect on ERK1/2 phosphorylation, together with 100 µM UTP. UTP provoked a reduction of ERK1/2 phosphorylation, although to a lesser extent compared to NECA. A forskolin concentration (30 nM), with no effect on ERK1/2 phosphorylation increased the effect of UTP, suggesting a synergism between cAMP and calcium in the regulation of ERK1/2 phosphorylation (Fig. 29).
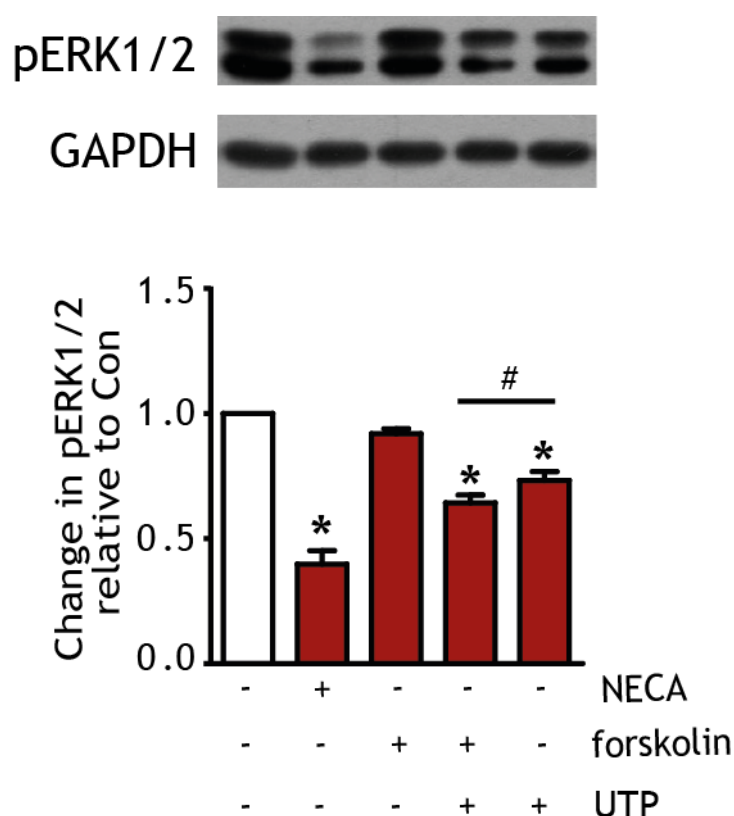
Figure 29: **Reduction of ERK1/2 phosphorylation by UTP compared to NECA.** The effect of 100 µM UTP was consistently lower than of 100 nM NECA. Addition of 30 nM forskolin to 100 µM UTP increased the UTP effect although forskolin at this concentration had no effect on its own. The Western blot shows a representative experiment, the columns represent mean values from n = 7 independent experiments (* $p < 0.001$, significantly different from untreated cells; # $p < 0.05$, UTP significantly different from UTP+forskolin).

Thus, cAMP and calcium act synergistically to reduce the ERK1/2 phosphorylation. However, their effect (30 nM forskolin combined with 100 µM UTP) was still lower than the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation. These data indicate that there might be an additional (unknown) contribution to a reduced phosphorylation of ERK1/2 by $A_{2B}AR$ activation.

4.8   Activation of A$_{2B}$ARs increases the CREB phosphorylation on S133

The cAMP pathway activates several transcription factors, including CREB, CREM, and ATF-1. Among them, CREB is the major effector of the cAMP pathway. CREB family members have been described as important transcription factors regulating cell proliferation and other nuclear responses (De Cesare et al., 1999). Endogenous A$_{2B}$ARs lead to cAMP accumulation and calcium mobilization in MDA-MB-231 cells. These signals are known to trigger the phosphorylation of CREB on S133. This phosphorylation event is required for CREB to activate for example the transcription of genes containing CREB binding sites (Dash et al., 1991; Gonzalez et al., 1991; Gonzalez and Montminy, 1989; Sheng et al., 1990; Sheng et al., 1991). We examined whether activation of A$_{2B}$AR leads to phosphorylation of CREB at S133. In this case, it is likely that the activation of A$_{2B}$AR increases the CREB-phosphorylation by stimulating both cAMP and Ca^{2+}. NECA, by stimulating cAMP and Ca^{2+} pathways, forskolin, which is an AC activator, and UTP, which is responsible for Ca^{2+} mobilization, all together induce the phosphorylation of CREB as assayed by Western blot (Fig. 30). Inhibition of PKA with H89 or PLC with U73122 reversed the increase of CREB phosphorylation by NECA. Futhermore, inhibition of phospatases using sanguinarine potentiated the increase in CREB phosphorylation. Additionally, the increase in CREB phosphorylation caused by NECA was not blocked by chelating the intracellular Ca^{2+} with BAPTA-AM (Fig. 31).
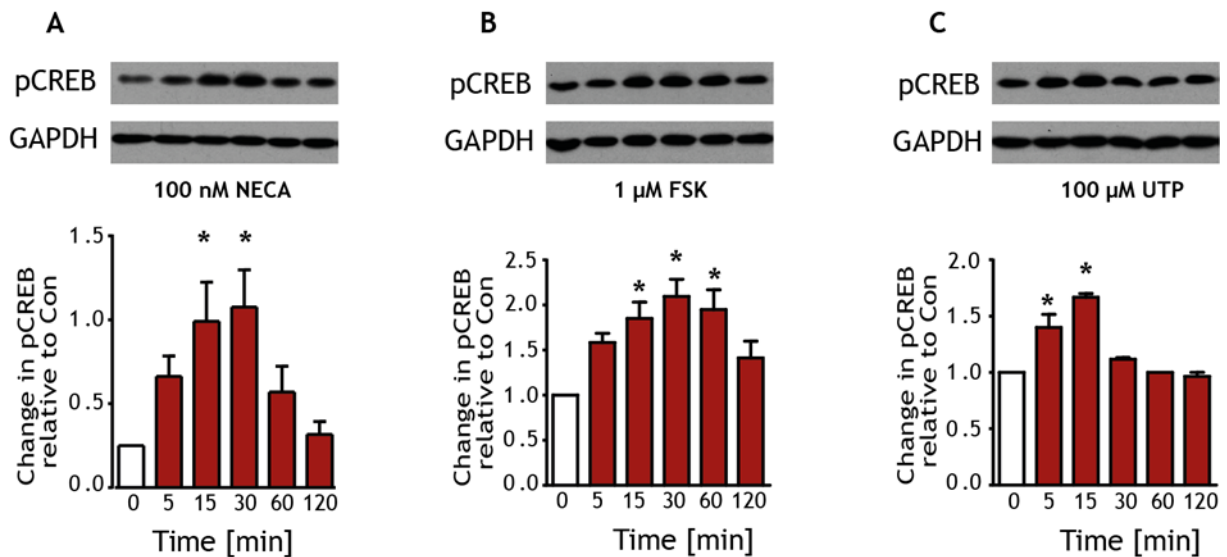
Figure 30: **CREB phosphorylation in MDA-MB-231 cells.** MDA-MB-231 cells were treated with 100 nM NECA (A), with 1 µM forskolin (B) or 100 µM UTP (C) for the indicated times, and then analyzed by Western blot analysis for pCREB. The Western blots show representative experiments, the columns represent mean values from n = 4 (A), 5 (B), and 3 (C) separate experiments, respectively (* $p < 0.01$ at 15 min (A); * $p < 0.001$ at 30 min (B); * $p < 0.001$ at 15 min (C), significantly different from untreated cells).
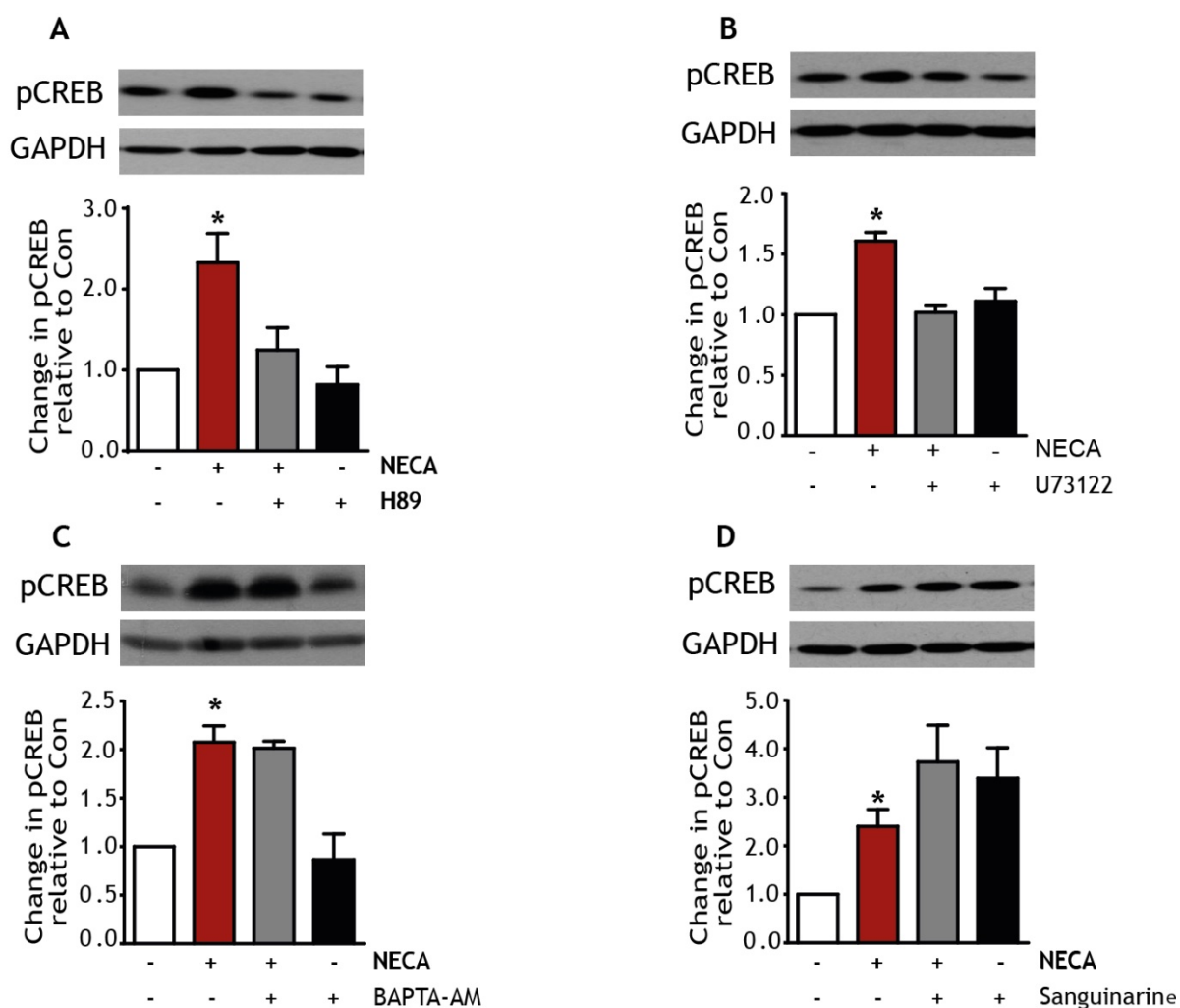
Figure 31: **CREB phosphorylation in MDA-MB-231 cells.** Preincubation (30 min) of MDA-MB-231 cells with 10 µM of the PKA inhibitor H89 (A), or with 10 µM of the PLC inhibitor U73122 (B) prevented the NECA effect on CREB phosphorylation. While incubation with 30 µM of the Ca^{2+}-chelator BAPTA-AM did not reverse the NECA-mediated CREB phosphorylation increase (C), 10 µM Sanguinarine, a phosphatase inhibitor, slightly potentiated the NECA effect (D). The Western blots show representative experiments, the columns represent mean values from n = 3 (A-C) independent experiments, respectively (* $p < 0.001$ (A); * $p < 0.01$ (B-C); * $p < 0.05$ (C) significantly different from untreated cells).

$A_{2B}AR$ induced CREB phosphorylation via a direct PKA-dependent mechanism. The increase of CREB activation by UTP probably reflects a calcium-dependent phosphorylation of CREB.

Upon activation of the $A_{2B}AR$ in MDA-MB-231 cells, we observed a reduction of ERK1/2 phosphorylation, as well as an increase of MKP-1 and MKP-2 protein expression. The MKP expression did not precede the reduction of ERK1/2 phosphorylation. Moreover, knocking down the MKPs did not affect the reduction of ERK1/2 phosphorylation. Therefore, the reduction of ERK1/2 phosphorylation can not be only attributed to

phosphatase activity. Based on our results we speculate that there may be another effector involved in the reduction of ERK1/2 phosphorylation downstream of the A$_{2B}$AR. c-Raf-1 and MEK1/2 are two kinases upstream of ERK1/2. Many studies have shown that PKA can phosphorylate and inhibit c-Raf-1, provoking a reduction of ERK1/2 phosphorylation. Therefore, we investigated the c-Raf-1/MEK1/2 signaling pathway in MDA-MB-231 cells.

4.9 Activation of A$_{2B}$AR reduces the phosphorylation of MEK1/2, a MAPK kinase

The lack of an effect of MKP-1 and MKP-2 silencing on reduction of ERK1/2 phosphorylation led us to investigate whether the c-Raf-1 and MEK1/2 upstream activators of the MAP kinases ERK1/2 may be involved. MEK1/2 belongs to the MAPKK family and is an important component of the MAPK cascade. MEK1/2 can be phosphorylated and activated by several upstream kinases including c-Raf-1. In turn the MAP kinase kinases (MEK1/2) phosphorylate and activate the MAPKs ERK1/2 (Chang and Karin, 2001). Several studies reported that the phosphorylation of two serine residues (S218 and S222) is required for MEK1/2 activation (Roskoski, 2012b; Zheng and Guan, 1993). To determine whether NECA can induce the inhibition of the protein kinase MEK1/2, the phosphorylation states of MEK1/2 were assessed in MDA-MB-231 cells. The effect of diverses activators on the amount of phospho-MEK1/2 was investigated. Compared to ERK1/2 phosphorylation, similar changes in the phosphorylation of MEK1/2 were observed in MDA-MB-231 cells. The A$_{2B}$AR stimulation provoked a transient reduction of MEK1/2 phosphorylation. Similarly, the AC activator forskolin reduced the MEK1/2 phosphorylation. However, in response to UTP, the MDA-MB-231 cells exhibited a different change in MEK1/2 phosphorylation when compared to NECA and FSK (Fig. 32).
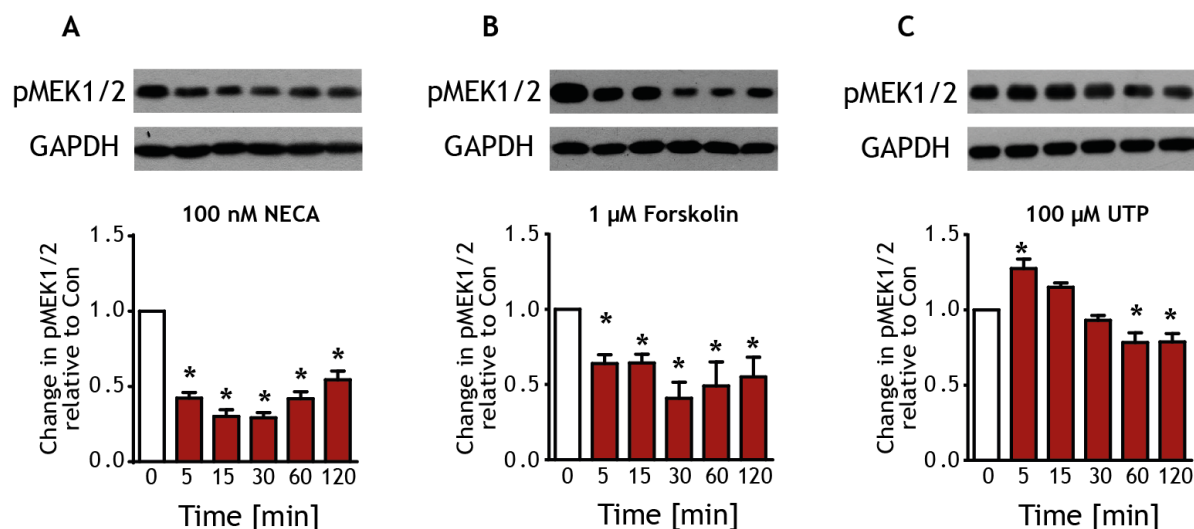
Figure 32: **Time dependent pMEK1/2 reduction by A₂ᵦAR activation in MDA-MB-231 cells.** MDA-MB-231 cells were incubated with 100 nM NECA (A), 1 µM Forskolin (B) or with 100 µM UTP (C) for the indicated times prior to determination of pMEK1/2. The insets show typical immunoblots representing pMEK1/2 (top) and GAPDH (bottom). Each column is the mean value of at least three independent experiments (* $p < 0.0001$ (A); * $p < 0.01$ (B); * $p < 0.01$ at 15 min, * $p < 0.05$ after 1 hour (C), significantly different from untreated cells).

Taken together, these results revealed that AC signaling appears to regulate the phosphorylation of MEK1/2. Increasing intracellular calcium mobilization failed to provoke a reduction of MEK1/2 phosphorylation. Because our results have implicated PKA in the reduction of ERK1/2 phosphorylation, we examined whether A₂ᵦAR mediated reduction of MEK1/2 phosphorylation was sensitive to PKA inhibition. The reduction of pMEK1/2 was reversed by pretreatment with 10 µM H89, a PKA inhibitor (Fig. 33A). Inhibition of PLC prevents the reduction of pMEK1/2 by NECA, too (Fig. 33B). CHX, the protein synthesis inhibitor, and BAPTA-AM, a Ca^{2+} chelator that block MKP-1/MKP-2 expression and antagonize the reduction of ERK1/2 phosphorylation in MDA-MB-231 cells, did not reverse the NECA-mediated reduction of pMEK1/2 (Fig. 34B-C). The reduction of pMEK1/2 is blocked in cells pretreated with sanguinarine, a phosphatase inhibitor. However, CHX alone reduced the basal phosphorylation of MEK1/2. These results suggest that *de novo* protein synthesis is not required for MEK1/2 inhibition by A₂ᵦAR stimulation (Fig. 34A).

A

pMEK1/2

GAPDH

| - | + | + | - | NECA |
| - | - | + | + | H89 |

B

pMEK1/2

GAPDH

| - | + | + | - | NECA |
| - | - | + | + | U73122 |
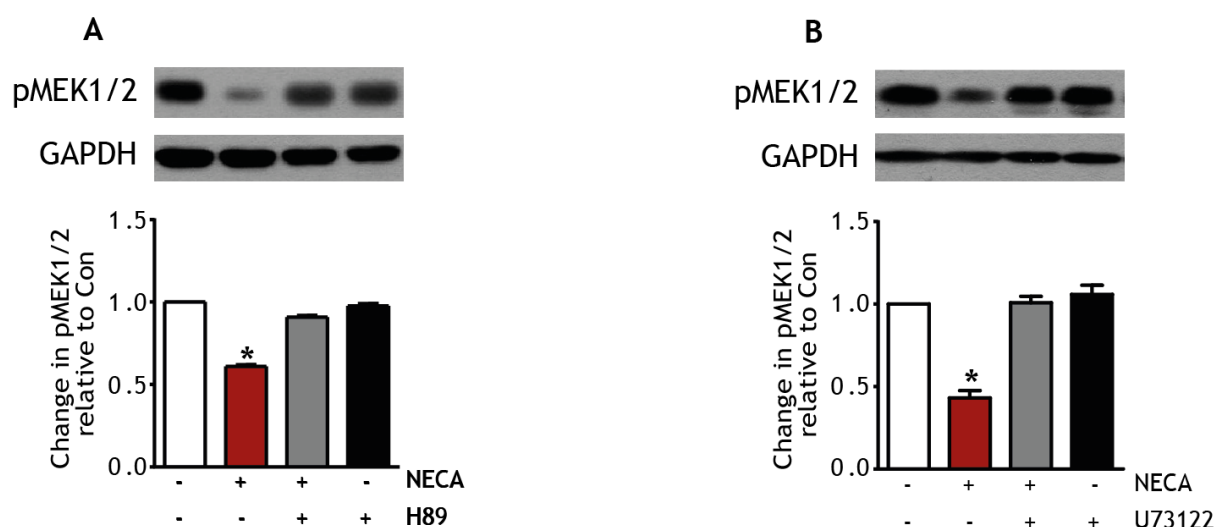
Figure 33: **A₂ᵦAR-mediated pMEK1/2 reduction in MDA-MB-231 cells is sensitive to the PKA inhibitor H89 and to PLC inhibitor U73122.** MDA-MB-231 cells were preincubated for 30 min with either 10 μM H89 (A) or 10 μM U73122 (B). Cells were then stimulated with 100 nM NECA for 30 min, and MEK1/2 phosphorylation was determined by Western blot analysis. Representative immunoblots of pMEK1/2 (top) and GAPDH (bottom) are shown. Each column is the mean value of n = 3(A) and 5(B) independent experiments, respectively (* $p < 0.0001$ significantly different from untreated cells).

A

pMEK1/2

GAPDH

| - | + | + | - | NECA |
| - | - | + | + | CHX |

B

pMEK1/2

GAPDH

| - | + | + | - | NECA |
| - | - | + | + | BAPTA-AM |

C

pMEK1/2

GAPDH

| - | + | + | - | NECA |
| - | - | + | + | Sanguinarine |
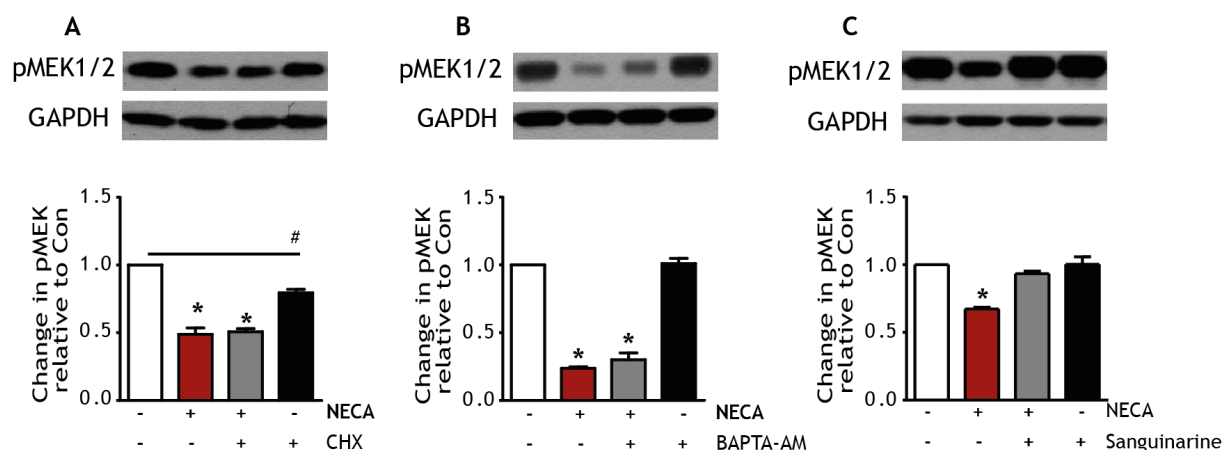
Figure 34: **A₂ᵦAR-mediated pMEK1/2 reduction in MDA-MB-231 cells is sensitive to sanguinarine but not to CHX and BAPTA-AM.** MDA-MB-231 cells were preincubated for 30 min with either 10 μg/ml CHX (A), 30 μM BPATA AM (B) or 10 μM sanguinarine (C). Cells were then stimulated with 100 nM NECA, and MEK1/2 phosphorylation was determined by Western blot analysis. Representative immunoblots of pMEK1/2 (top) and GAPDH (bottom) are shown. The data are normalized to the non-stimulated responses and each bar is the mean of at least three independent experiments (* $p < 0.0001$ (A-B), # $p < 0.01$ (A), * $p < 0.001$ (C) significantly different from untreated cells).

4.10 Activation of A$_{2B}$AR regulates the phosphorylation of the MAPKKK c-Raf-1

Having observed the reduction of the MAPK kinase (MKK or MEK1/2) phosphorylation, the next logical step was to examine the involvement of c-Raf-1 in this process. c-Raf-1 has been shown to be a major upstream activator of MEK1/2 and is involved in the transmission of developmental and proliferative signals generated by receptor and non-receptor tyrosine kinases. Thus, c-Raf-1 constitutes a point of regulation in the MAP kinase pathway. However, the mechanism(s) of c-Raf-1 regulation are diverse. In essence, phosphorylation plays a key role in the regulation of c-Raf-1 activity. Phosphorylation of c-Raf-1 is important for both positive and negative regulation of biological activity. Five activating phosphorylation sites within its kinase domain have been identified, with serine 338 (S338) being one of them (Hamilton et al., 2001; King et al., 1998). PKA phosphorylates three sites in c-Raf-1 to block its activation when cyclic AMP levels are elevated (Dumaz and Marais, 2003). Serine 259 (S259) is the major target for c-Raf-1 inhibition by cAMP dependent kinase PKA (Dhillon et al., 2002; Zimmermann and Moelling, 1999). Activated c-Raf-1 modulates the phosphorylation state of MEK1/2, which in turn activates the MAPK ERK1/2. This means that changes in the phosphorylation levels of c-Raf-1 could influence the ERK1/2 phosphorylation levels. To further investigate the importance of c-Raf-1/MEK1/2 in the mediation of A$_{2B}$AR-induced reduction of ERK1/2 phosphorylation, we examined whether A$_{2B}$AR stimulation could change the phosphorylation levels of c-Raf-1, the MAPKK kinase (Fig. 35).
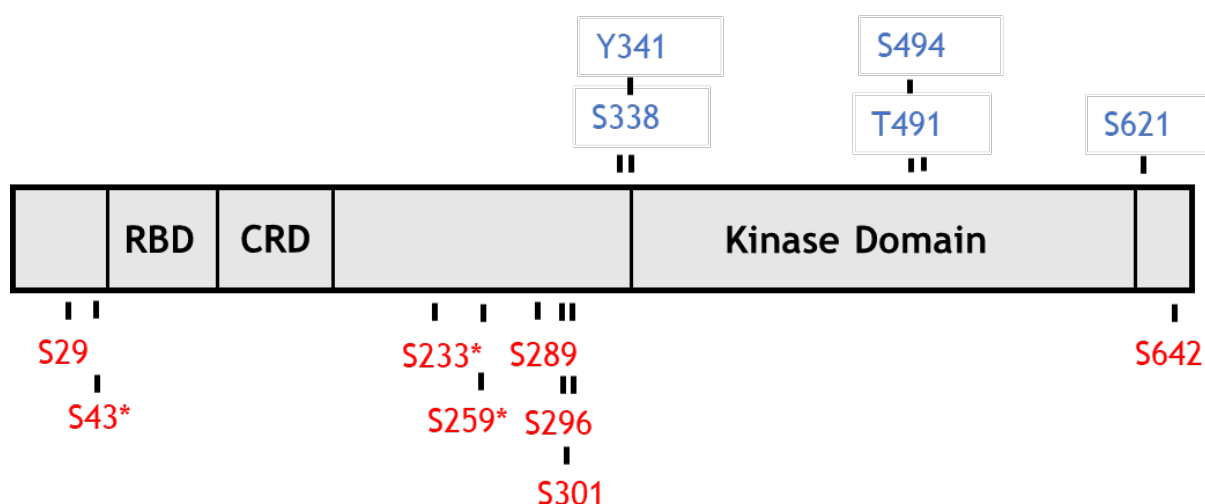
Figure 35: **Schematic representation of Raf-1 phosphorylation sites (modified from Dumaz and Marais, 2005b).** Phosphorylation of residues marked in blue is required for Raf-1 activity, whereas those in red block its activation. Residues marked with an asterisk are phosphorylated by PKA. (Abbreviations: RBD, Ras binding domain; CRD, cystein-rich domain)

We examined the effect of NECA on the phosphorylation-kinetics of endogenous c-Raf-1 on S338 and S259. c-Raf-1 was phosphorylated on S338 (Fig. 36) and weakly on S259 in untreated cells, as shown in (Fig. 39). NECA induced a decrease of c-Raf-1 phosphorylation on S338 after 5 min. A similar trend was observed after stimulation of AC with forskolin (Fig. 36A-B). By contrast, UTP did not affect the phosphorylation on S338. Similar to MEK1/2 phosphorylation, no discernible difference in c-Raf-1 phosphorylation on S338 was observed in cells treated with UTP (Fig. 36C).
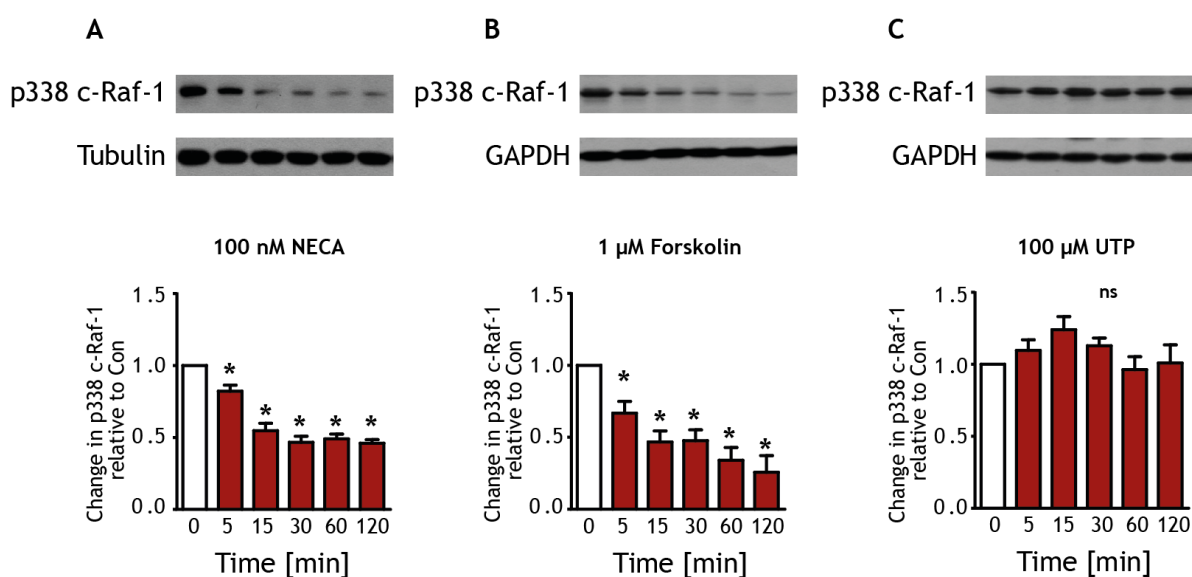
Figure 36: **Phosphorylation of S338 of c-Raf-1 is reduced by AC activation.** A time-dependent inhibition of phosphorylation of c-Raf-1 at S338 is observed by stimulation of the $A_{2B}AR$ with NECA (A). A similar effect was seen with forskolin (B) while UTP showed no reduction of S338 phosphorylation (C). The Western blots show representative experiments, the columns represent mean values from n = 5 (A), 4 (B) and 5 (C) independent experiments, respectively (* $p < 0.0001$, 15min (A); * $p < 0.01$, 15min (B); significantly different from untreated cells).

To determine whether PKA activation is involved in the reduction of c-Raf-1 phosphorylation on S338, the cells were pretreated for 30 min with H89, followed by 30 min incubation with NECA or forskolin. Under these conditions, the PKA inhibitor H89 reversed NECA- or forskolin-mediated reduction of c-Raf-1 phosphorylation on S338, suggesting that activation of AC/cAMP and of the downstream cAMP effector PKA is critical for the phosphorylation of c-Raf-1 on S338. In contrast, inhibition of PLC by 10 µM U73122 did not reverse the reduction of c-Raf-1 phosphorylation on S338. To our surprise, the presence of U73122 alone reduced the phosphorylation of c-Raf-1 on S338 (Fig. 37). Incubation with the protein synthesis inhibitor CHX, the calcium chelator BAPTA-AM, and the phosphatase inhibitor sanguinarine did not antagonize the reduction of c-Raf-1 phosphorylation at S338 (Fig. 38).
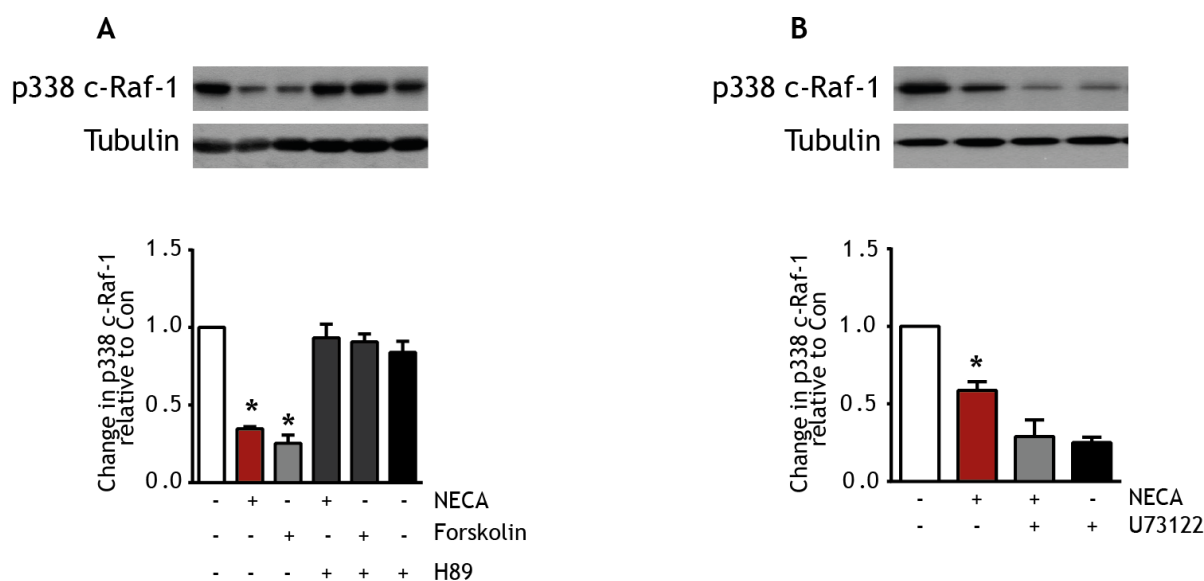
Figure 37: **A₂ʙAR activation mediates the reduction of p338 c-Raf-1 in a PKA-dependent manner.** Preincubation (30 min) of MDA-MB-231 cells with 10 µM of the PKA inhibitor H89 (A) prevented the NECA and the forskolin effect on p338 c-Raf-1 while inhibition of PLC with U73122 showed no effect (B). The Western blots show representative experiments, the columns represent mean values from n = 3 (A) and 5 (B) independent experiments, respectively (* $p < 0.0001$ (A); * $p < 0.01$ (B) significantly different from untreated cells).
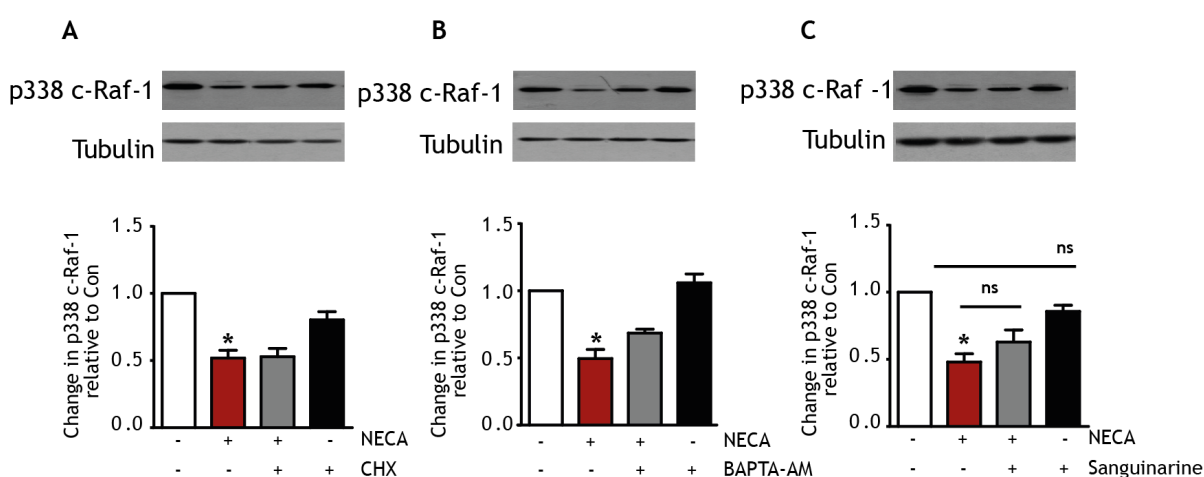
Figure 38: **A₂ʙAR-mediated p338 c-Raf-1 reduction did not require *de novo* protein synthesis, nor did BAPTA-AM or sanguinarine affect this reduction.** MDA-MB-231 cells were preincubated for 30 min with 10 µg/ml CHX (A), 30 µM BAPTA-AM (B), or with 10 µM sanguinarine (C) prior to stimulation with 100 nM NECA for 30 min. c-Raf-1 phosphorylation was analyzed by immunoblotting. Representative immunoblots of p338 c-Raf-1 (top) and tubulin (bottom) are shown. The data are normalized to the non-treated cells and each bar is the mean values from n = 4 (A), 5 (B) and 4 (C) independent experiments, respectively (* $p < 0.001$, significantly different from untreated cells).

These findings suggest that activation of $A_{2B}AR$ in MDA-MB-231 cells may lead to activation of an AC/PKA dependent effector, which in turn reduces the c-Raf-1 phosphorylation on S338. As opposed to S338 phosphorylation, S259 was weakly phosphorylated in untreated cells, but then phosphorylation increased following incubation with all tested activators (NECA, forskolin, cAMP, or UTP, Fig. 39). Together, these results demonstrate that stimulation of $A_{2B}AR$ leads to the inhibition of c-Raf-1 through an increase of the major PKA phosphorylation site S259, and the reduction of the c-Raf-1-activating phosphorylation site S338. The activation of $A_{2B}AR$ in MDA-MB-231 cells negatively regulates enzymes upstream of ERK1/2, namely MEK1/2 and c-Raf-1, thus providing an additional pathway for the reduction of ERK1/2 phosphorylation.
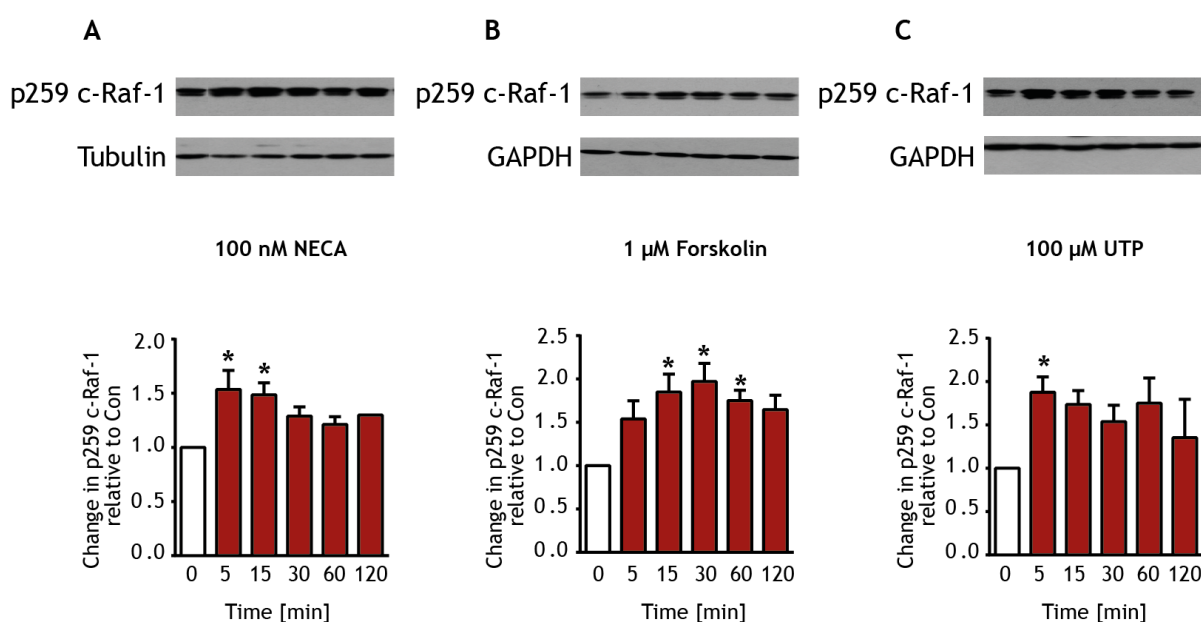
Figure 39: **Phosphorylation of c-Raf-1 on S259 is induced by AC activation and by Ca^{2+} release.** A time-dependent increase of phosphorylation of c-Raf-1 on S259 is observed by stimulation of the $A_{2B}AR$ with NECA (A). A similar effect was seen with forskolin (B) and UTP (C) The Western blots show representative experiments, the columns represent mean values from n = 5 (A), 10 (B) and 4 (C) independent experiments, respectively (* $p < 0.01$ (A-B); * $p < 0.05$ (C) significantly different from untreated cells).

4.11 Effect of A$_{2B}$ARs activation on the stress activated MAPK

4.11.1 JNK

The MAPK pathways also include the stress activated protein kinases JNK and p38. cAMP has been shown to regulate the MAP kinases in a cell- and stimulus-dependent manner. Since A$_{2B}$AR activation in MDA-MB-231 cells provoked an increase of cAMP, we investigated the effect of A$_{2B}$AR activation in MDA-MB-231 cells on JNK. After stimulation with NECA, immunoblotting analysis revealed that phosphorylation of JNK at Thr183 and Tyr185, which are important for JNK activation, was significantly reduced in a time-dependent manner. Treatment with forskolin or cAMP-AM also led to a similar reduction of JNK phosphorylation (Fig. 40).
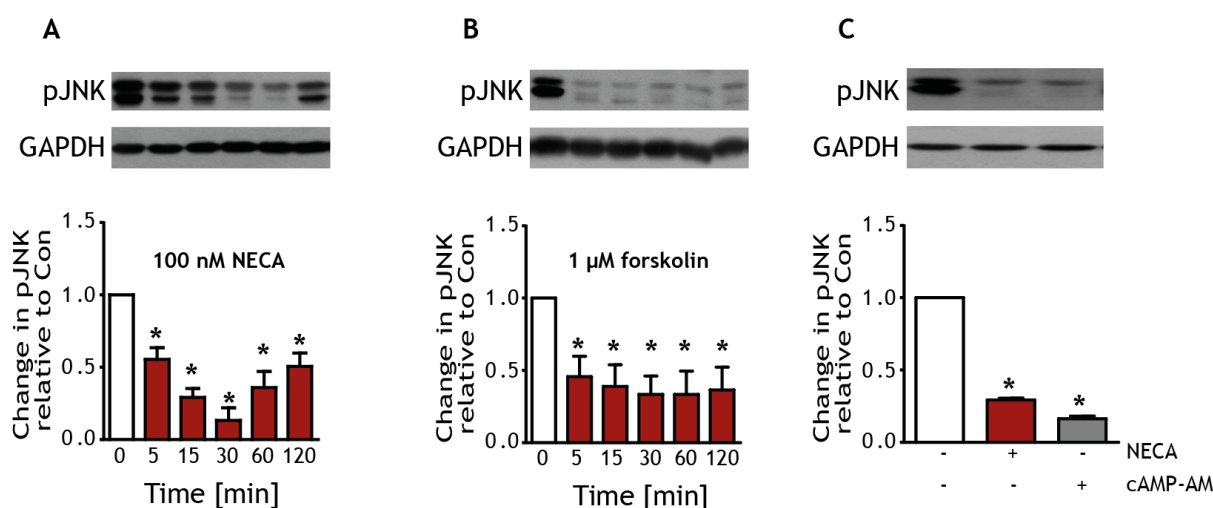
Figure 40: **A$_{2B}$AR-mediated reduction of pJNK was mimicked by compounds increasing cAMP production.** MDA-MB-231 cells were treated for different time periods with 100 nM NECA (A) or with 1 µM forskolin (B) and with 100 µM cAMP-AM ("caged cAMP", C) for 30 min. The Western blots show representative experiments, the columns represent mean values from n = 5 (A), 4 (B), and 3 (C) independent experiments, respectively (* p < 0.001(A; C), * p < 0.05(B), significantly different from untreated cells).

Likewise, increasing the intracellular calcium with UTP reduced JNK phosphorylation (Fig. 41A). The PKA inhibitor H89 and the PLC inhibitor U73122 reversed the negative effect of NECA on JNK phosphorylation in MDA-MB-231 cells. A$_{2B}$AR receptor activation reduced JNK phosphorylation in a PKA-and PLC dependent manner (Fig. 41B-C).
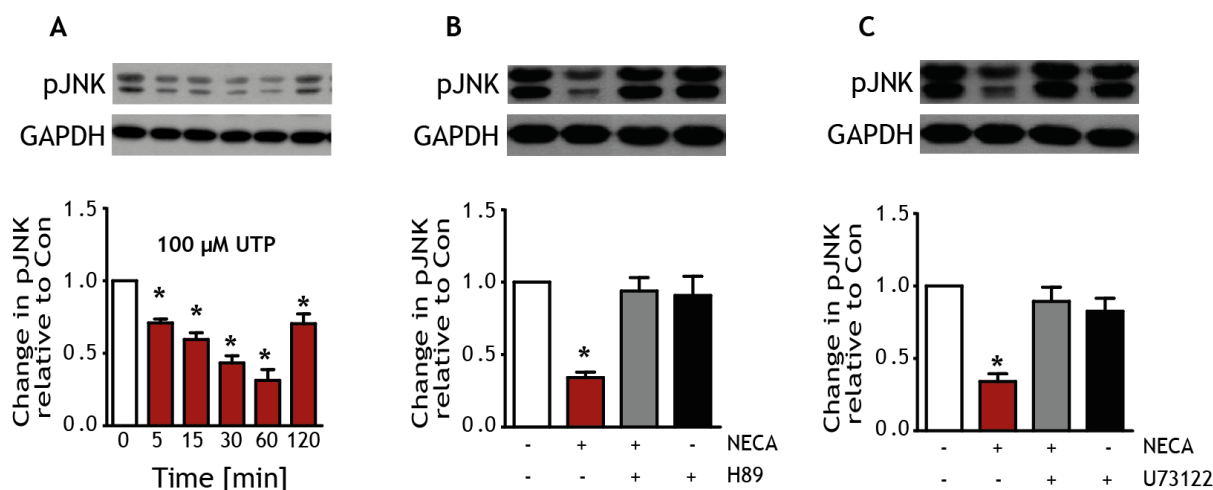
Figure 41: **A₂ʙAR-mediated reduction of pJNK is PKA and Ca²⁺ dependent.** Increasing intracellular Ca²⁺ mobilization with 100 µM UTP reduced pJNK (A), 30 min preincubation with 10 µM PKA inhibitor H89 (B) or 10 µM PLC inhibitor U73122 (C) prior to 100 nM NECA treatment for 30 min also blocked the NECA-mediated reduction of pJNK. The Western blots show representative experiments, the columns represent mean values from n = 4 (A), 6 (B), and 5 (C) independent experiments, respectively (* p < 0.0001, 30 min (A), * p < 0.0001 (B-C) significantly different from untreated cells.

Like ERK1/2 phosphorylation, new protein synthesis was apparently required for the inhibition of JNK, as the A₂ʙAR-mediated reduction of JNK phosphorylation was reversed in the presence of CHX (Fig. 42A). Presumably, the suppression of JNK was mediated by a newly synthesized inhibitor upon stimulation of the A₂ʙAR. The MAPK phosphatases MKP-1/MKP-2 might be candidates since their expression increased in MDA-MB-231 cells when exposed to cAMP or calcium elevating agents. Consequently, the calcium chelator BAPTA-AM and the MKP inhibitor sanguinarine reversed the reduction of JNK phosphorylation, but also affected the basal JNK phsohorylation significantly (Fig. 42B-C). Furthermore, we found that the knockdown of MKP proteins led to partial inhibition of the A₂ʙAR-mediated reduction of JNK phosphorylation (Fig. 43).
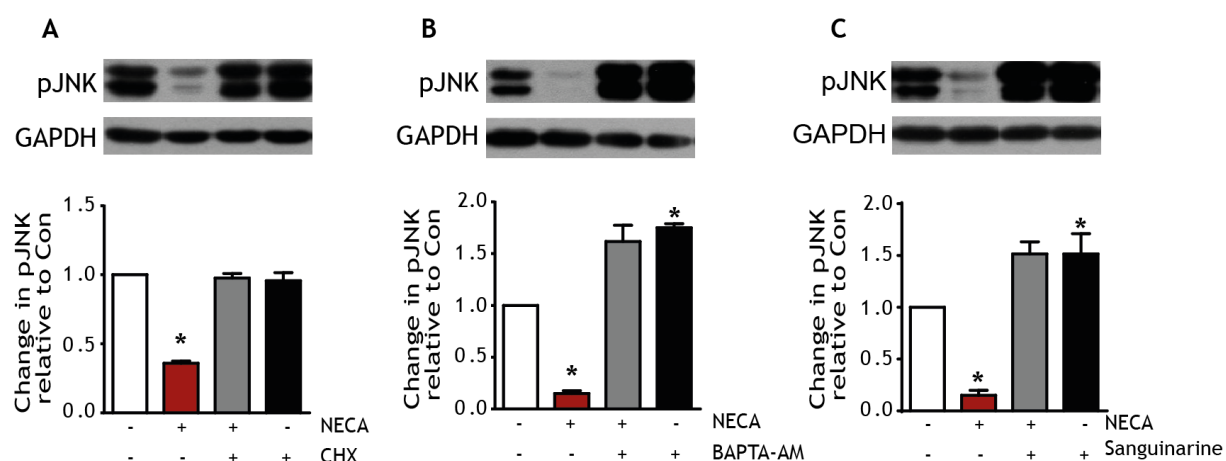
Figure 42: **A$_{2B}$AR-mediated reduction of JNK phosphorylation required *de novo* protein synthesis** MDA-MB-231 cells were treated for 30 min with 10 µg/ml CHX (A), 30 µM BAPTA-AM or with 10 µM sanguinarine (C) before 30 min treatment with 100 nM NECA. JNK phosphorylation was analyzed by immunoblotting. The Western blots show representative experiments, the columns represent mean values from n = 3 (A), 4 (B), and 3 (C) independent experiments, respectively (* p < 0.0001 (A, B), * p < 0.01; *p < 0.05 (NECA, sanguinrine treated, respectively, C), significantly different from untreated cells).

-	+	+	-	+	-	+	-	+	-	NECA
-	-	+	+	+	+	-	-	-	-	MKP-2 siRNA
-	-	-	-	+	+	+	+	-	-	MKP-1 siRNA
-	-	-	-	-	-	-	-	+	+	non target siRNA
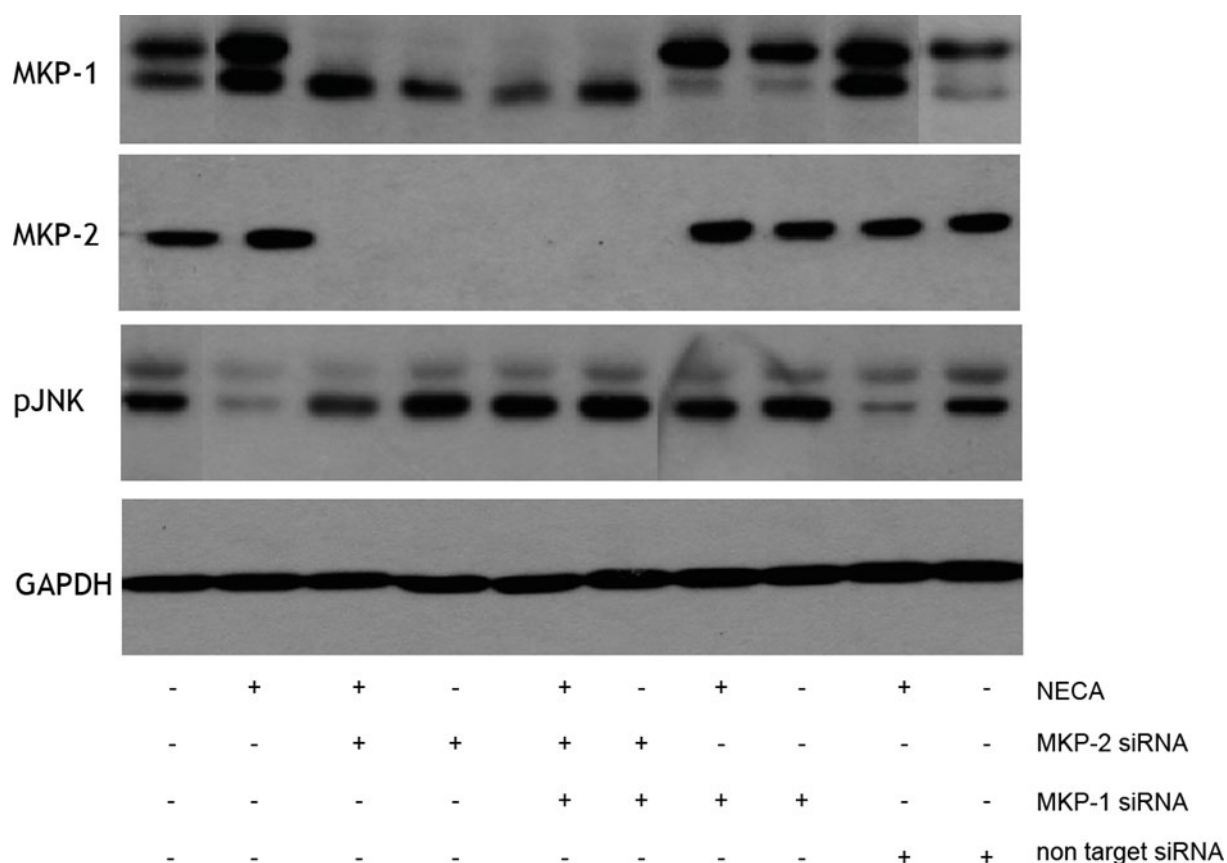
Figure 43: **The knockdown of MKP proteins reversed the A$_{2B}$AR-mediated reduction of pJNK.** MKPs expression after NECA treatment in MDA-MB-231 cells transfected with 50 nM siRNA targeting either MKP-1 or MKP-2 or 50 nM with non target siRNA for 48 hours then stimulated with or without 100 nM NECA for 30 min and then subjected to western blot analysis. Representative immunoblots showing the protein levels of MKP-1, MKP-2, pJNK and GAPDH (bottom panel) is included to demonstrate loading consistency.

A$_{2B}$AR activation has the potential to reduce the phosphorylation of MAPK, ERK1/2, and JNK through cAMP- or calcium- induced upregulation of MKP-1/MKP-2 expression. If the reduction of the JNK required only the newly synthetized phosphatase, we expect the effect to be slow (hours). Like for ERK1/2 phosphorylation, the reduction of JNK phosphorylation is observed within minutes. These results suggested an additional effector being involved in the A$_{2B}$AR effect.

4.11.2   p38 MAPK

Similar to other MAPK pathways, p38 MAPK is activated as a result of phosphorylation at specific sites by an upstream family of MAPKKK and MAPKK. Consequently, the MAPKKs which lie upstream of p38 MAPK selectively phosphorylate p38 MAPK at Thr180 and Tyr182. Several studies have reported PKA-dependent activation of p38 MAPK in a variety of cells, including CHO, colon cancer cells, and adult mouse cardiomyocytes. However, activation of AC has also been shown to inhibit p38 MAP kinase activation in e.g. HUVEC cells and in thymocytes. Recently, an alternative p38 activation mechanism that bypasses MKK-dependent MAPK phosphorylation was described, involving autophosphorylation of the p38 isomer. The noncanonical phosphorylation of p38 is induced by direct binding of TAB1 (TAK-1-binding protein) to the C-terminal domain of p38 (DeNicola et al., 2013; Ge et al., 2002; Zhou et al., 2006). In comparison to the ERK1/2 and JNK pathways, $A_{2B}AR$ stimulation seems to have the opposite effect on p38 MAPK activity in MDA-MB-231 cells. Pretreatment with NECA resulted in an increase of p38 MAPK phosphorylation. NECA-stimulated p38 phosphorylation was rapid, reaching its peak within 5 minutes. Like NECA treatment, incubation of MDA-MB-231 cells by UTP also led to an increase of p38 phosphorylation. To our surprise, incubation with forskolin did not increase the p38 phosphorylation, but promoted a significant reduction of p38 phosphorylation after 1 hour (Fig. 44).
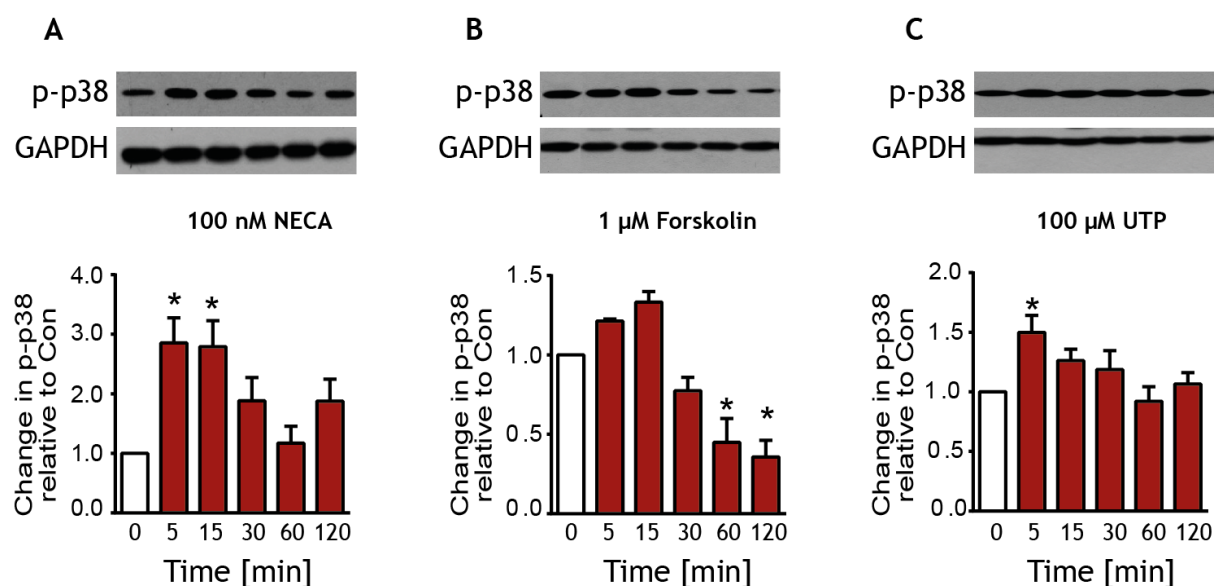
Figure 44: **A$_{2B}$AR-mediated p38 MAPK phosphorylation required Ca^{2+}.** MDA-MB-231 cells were exposed to NECA (A), FSK (B) or UTP (C) for the indicated times. The top panels show representative Western blots of phosphorylated p38 MAPK (p-p38) with GAPDH used as a loading control. The columns represent mean values from n = 7 (A), 3 (B), and 5 (C) independent experiments, respectively (* $p < 0.01$ (A, B), * $p < 0.001$ (C), significantly different from untreated cells).

To determine if A$_{2B}$AR activates p38 through the canonical three-tiered kinase cascade, we used the p38 inhibitor SB203580, which specifically inhibits the catalytic activity of p38 without blocking p38 phosphorylation mediated by upstream kinases (Davies et al., 2000; DeNicola et al., 2013; Ge et al., 2002). We reasoned that pretreatment with SB203580 would block the autophosphorylation, since it requires the p38 catalytic function. After incubation of MDA-MB-231 cells with SB203580, NECA was no longer able to increase the phosphorylation of p38 (Fig. 45). Our results show that inhibition of p38 activity prevents the increase of phosphorylation. We concluded that A$_{2B}$AR activation increases p38 phosphorylation through autophosphorylation.
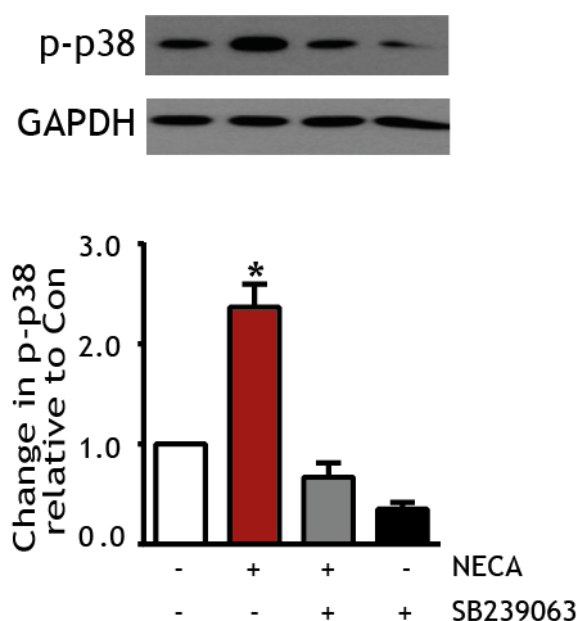
Figure 45: **A$_{2B}$AR activation induced p38 MAPK autophosphorylation.** MDA-MB-231 cells were preincubated with 10 µM SB239063 for 30 min, and then exposed to NECA (100 nM) for 10 min. NECA (100 nM) increased p38 MAPK phosphorylation after 10 min and this increase was prevented in MDA-MB-231 cells pretreated by 10 µM SB239063 (SB) for 30 min. The Western blots show representative experiments, the columns represent mean values from n = 3 independent experiments, respectively (* p < 0.05, significantly different from untreated cells).

4.12 Activation of the β-adrenergic receptor in MDA-MB-231 cells

We wanted to examine whether other G$_s$-coupled receptors might trigger similar pathways as the ones seen for the A$_{2B}$AR in MDA-MB-231 cells. For this purpose, MDA-MB-231 cells were stimulated with 100 nM isoproterenol (ISO), a nonselective β-AR agonist. We observed a time dependent reduction of ERK1/2 phosphorylation after stimulation of the endogenous β-AR. Furthermore, ISO triggered an increase of CREB phophorylation. The increase of CREB phosphorylation suggests that β-AR mediate the activation of the AC/cAMP/PKA pathways. Additionally, treatment with ISO led to calcium mobilization (Fig. 46).
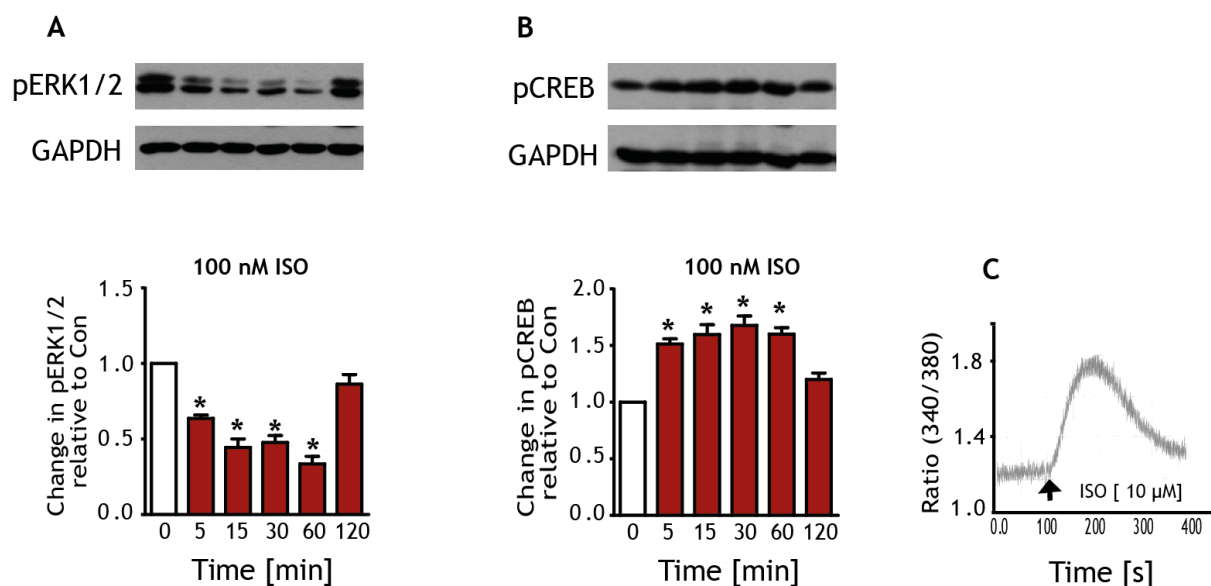
Figure 46: **β-Adrenergic receptor activation in MDA-MB-231 cells.** Cells were incubated with 100 nM ISO for indicated periods of time. Analysis of pERK1/2 reduction (A) and pCREB activation (B) was performed concomitantly in the same samples. The data were normalized to the level of protein phosphorylation at time (0). (A, B) showed significant changes in the amount of pERK1/2 (n = 6, A) and of pCREB (n = 6, B) (* $p < 0.0001$, significantly different from untreated cells). The Ca^{2+} trace show one representative experiment (C).

Similar to $A_{2B}AR$, the β-AR-mediated reduction of ERK1/2 phosphorylation implies PKA-, PLC- and Ca^{2+}-dependent pathways. Also, *de novo* protein synthesis seems to be involved (Fig. 47).
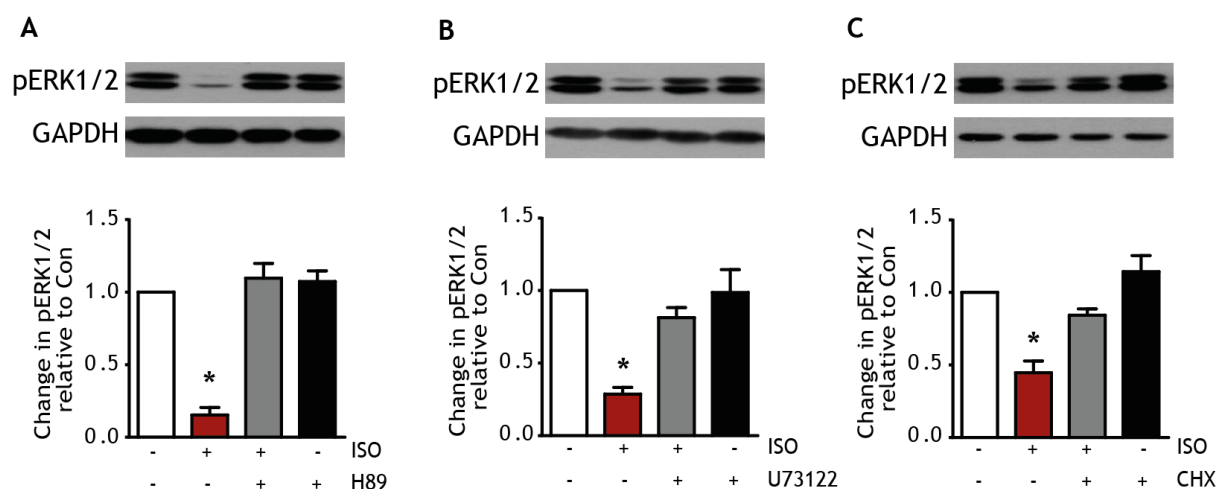
Figure 47: Preatreatment of MDA-MB-231 for 30 min with 10 µM H89 (A), 10 µM U73122 (B), and 10 µg/ml CHX (C) followed by 30 min incubation with 100 nM ISO reversed the reduction of ERK1/2 phosphorylation. The Western blots show representative experiments. The columns show data from n = 3 independent experiments, respectively (* p < 0.001(A), * p < 0.01(B-C), significantly different from untreated cells).

Consequently, an increase of MKP-1/MKP-2 expression was observed. Furthermore, Ca^{2+} chelating and the MKP inhibitor sanguinarine reversed the ISO-mediated reduction of ERK1/2 phosphorylation (Fig. 48).
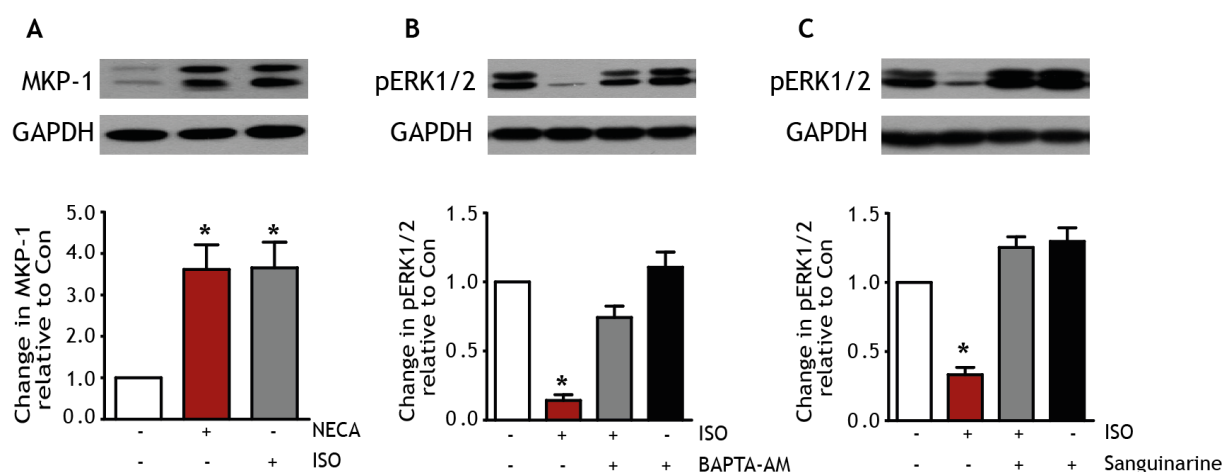
Figure 48: (A) Incubation of MDA-MB-231 cells with 100 nM ISO mimicked the NECA-mediated increase of MKP-1 and MKP-2 expression. Preatreatment of MDA-MB-231 for 30 min with 30 µM BAPTA-AM (B) 10 µM sanguinarine (C) followed by 30 min incubation with 100 nM ISO reversed the reduction of pERK1/2. The Western blots show representative experiments. The columns show data from n = 6 (A), 3 (B-C) independent experiments, respectively (* p < 0.01(A), * p < 0.001(A-B), significantly different from untreated cells).

In comparison to untreated cells, ISO-treated cells show a reduction of S338 phosphorylation of c-Raf-1 (Fig. 49A). Also, a time dependent reduction of JNK phosphorylation was observed (Fig. 49B).

A

B
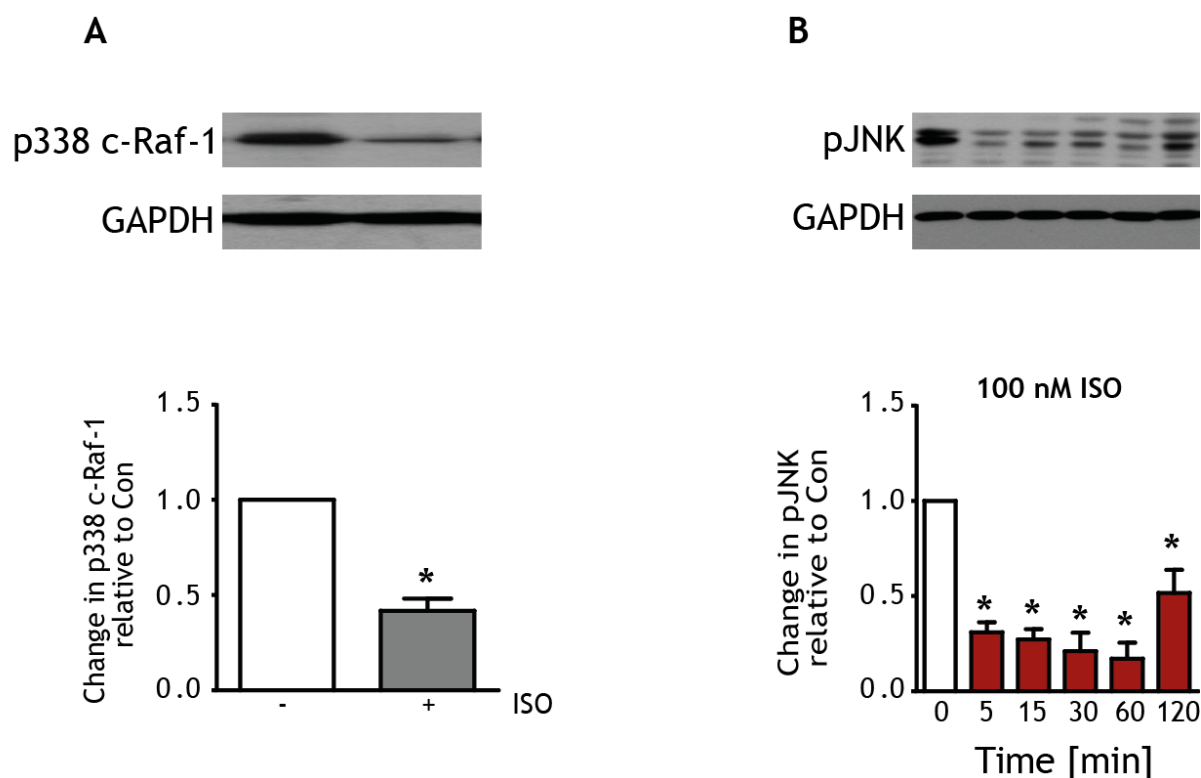
Figure 49: MDA-MB-231 cells were pretreated with 100 nM ISO for 30 min (A) or for the indicated times (B). Cell lysates were blotted with antibodies against p338 c-Raf-1 (A) and pJNK (B). The Western blots show representative experiments, the columns represent mean values of n = 5 (A), 4 (B), independent experiments, repectively (* $p < 0.001$, significantly different from untreated cells).

4.13 Functional selectivety of A$_{2B}$AR ligands

The MDA-MB-231 breast cancer cells express only A$_{2B}$AR as unique adenosine receptor. One of the most questionable points is that many studies on biased ligands were performed in cells where the receptor of interest is transfected with high expression levels. Furthermore, the receptor density is a well-known determinant of ligand efficacy. Therefore, MDA-MB-231 cells provide an adequate tool to study the functional selectivity of ligands at endogenous A$_{2B}$ARs. Different ligands may emphasize the activation of different signaling pathways via the same receptor. We investigated whether AR agonists may selectively engage in one signaling pathway, while sparing other responses linked to the A$_{2B}$AR. For this purpose, we selected the

nonselective AR agonists NECA und PHPNECA, the $A_{2B}AR$ agonist DPA23, and the $A_{2A}AR$ agonist UK-432097 as adenosine-derived nucleoside agonists. In addition, the nonnucleoside agonist LUF6210, a selective $A_{2B}AR$ agonist, was selected to investigate their functional activity in AC stimulation, calcium mobilization, and reduction of ERK1/2 phosphorylation. Membrane-binding or AC-studies were undertaken in CHO cell lines stably expressing the human $A_{2B}AR$ to determine the affinity and the potency of the ligands in the same cellular background. The results are summarized in Table 3 and show that all compounds bind to the $A_{2B}AR$. The nonnucleoside agonist LUF6210 showed similar affinity to NECA, but its affinity was 4- and 28-fold higher than the one exhibited by DPA23 and UK432097, respectively. All tested agonists demonstrated AC activation. LUF6210 and DPA23 were found to be the most potent agonists, whereas UK-432097, PHPNECA, and NECA were active at concentrations in the micromolar range. The order of potency is DPA23=LUF6210 >UK432097=PHPNECA=NECA.

Table.3: Affinity of the several ligands at $A_{2B}AR$ (K_i values in nM) and agonist potency for AC activation via $A_{2B}AR$ in CHO cells (EC_{50} values in nM; with 95% confidence intervals in parentheses).

$A_{2B}AR$ in CHO cells		
Compound	Binding (K_i in nM)	AC (EC_{50} in nM),(95% confidence intervals)
NECA	330 ($\pm$60)	2,360 (1,860-3,000)
PHPNECA	N.D.	1,110 (470-2,600)
DPA23	797	75.4 (59.7-95.2)
UK-432097	6368	617 (508-748)
LUF6210	228	115 (77.6-172)

In MDA-MB-231 cells, all ligands were studied in three different functional assays: AC-activation, Ca^{2+} mobilization and MAPK (ERK1/2) phosphorylation. Concentration-dependent experiments were performed to determine the EC_{50}/IC_{50} of the AR agonists. The experiments were monitored as described in the Methods section.

Stimulation of the MDA-MB-231 cells with the diverse agonists leads to an increase of cAMP (Fig. 50).
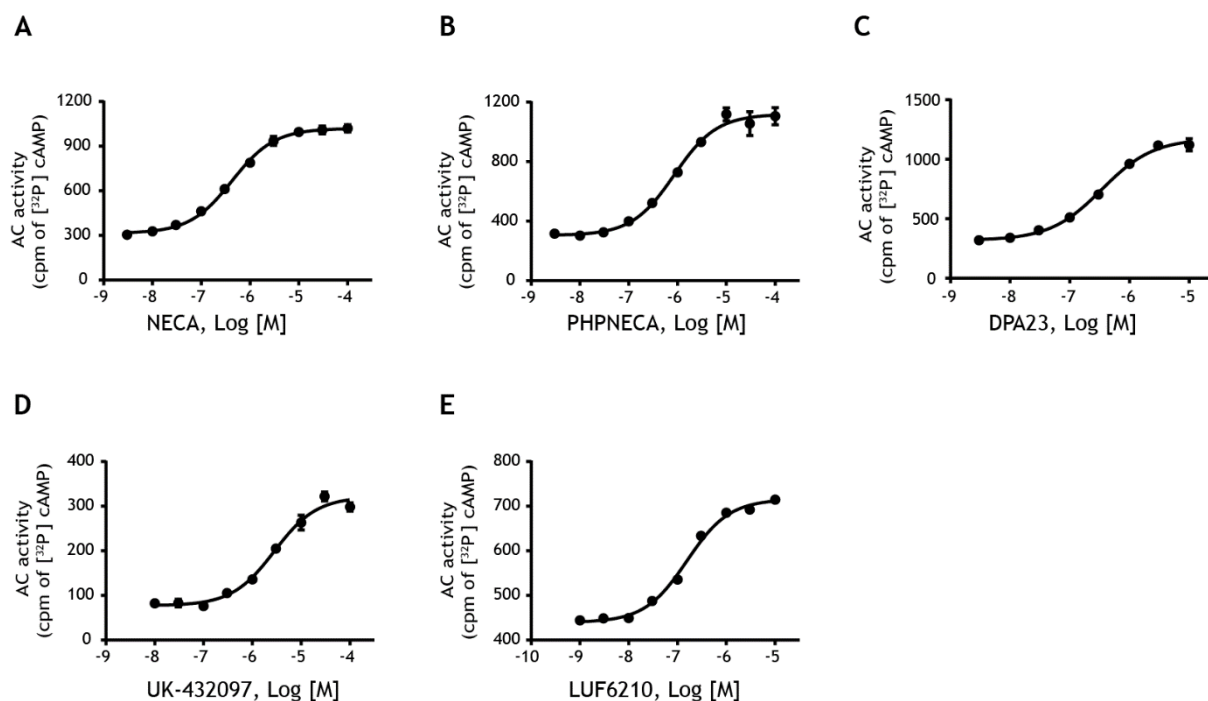
Figure 50: **Concentration-response curves for agonist-mediated AC activation in MDA-MB-231 membrane preparation.** The curves show the result of a representative experiment (mean values of determinations in duplicate) performed at least three times, giving EC_{50} of 427 (375-486) (A), 857 (656-1,119) (B), 344 (286-416) (C), 2,819 (2,052-3,872) (D) and 155 (104-234) (E) in nM with 95% confidence intervals in parentheses.

The dose dependency of the activation of AC by NECA and the other agonists was determined (Fig. 50). The EC_{50} values of the $A_{2B}AR$ agonists for the AC activation are summarized in Table 4. The half-maximal stimulation of AC by NECA occured at 427 nM (95% CI: 375-486 nM). LUF6210 and DPA23 were found to be as potent as NECA, while PHPNECA and UK-432097 were active in the micromolar range, with UK-432097 being the least potent. The rank order of potency was LUF6210 = DPA23 = NECA $\geq$ PHNECA $\geq$ UK-432097.

Table 4: Agonist potency for AC activation via A_{2B}ARs in MDA-MB-231 cell membranes (EC_{50} values in nM with 95% confidence intervals in parentheses).

Compound	AC (EC_{50} in nM) (95% confidence intervals)
NECA	427(375-486)
PHPNECA	857(656-1,119)
DPA23	344(286-416)
UK-432097	2,819(2,052-3,872)
LUF6210	155(104-234)

The nonnucleoside agonist LUF6210 acts as a partial agonist in AC activation. LUF6210 had an E_{max} of 40% compared to the full agonist NECA. The efficacy of the other agonists was similar to NECA. The rank order of agonist efficacy in the AC-assay was PHPNECA=NECA=DPA23>UK-432097>>LUF6210 (Fig. 51).
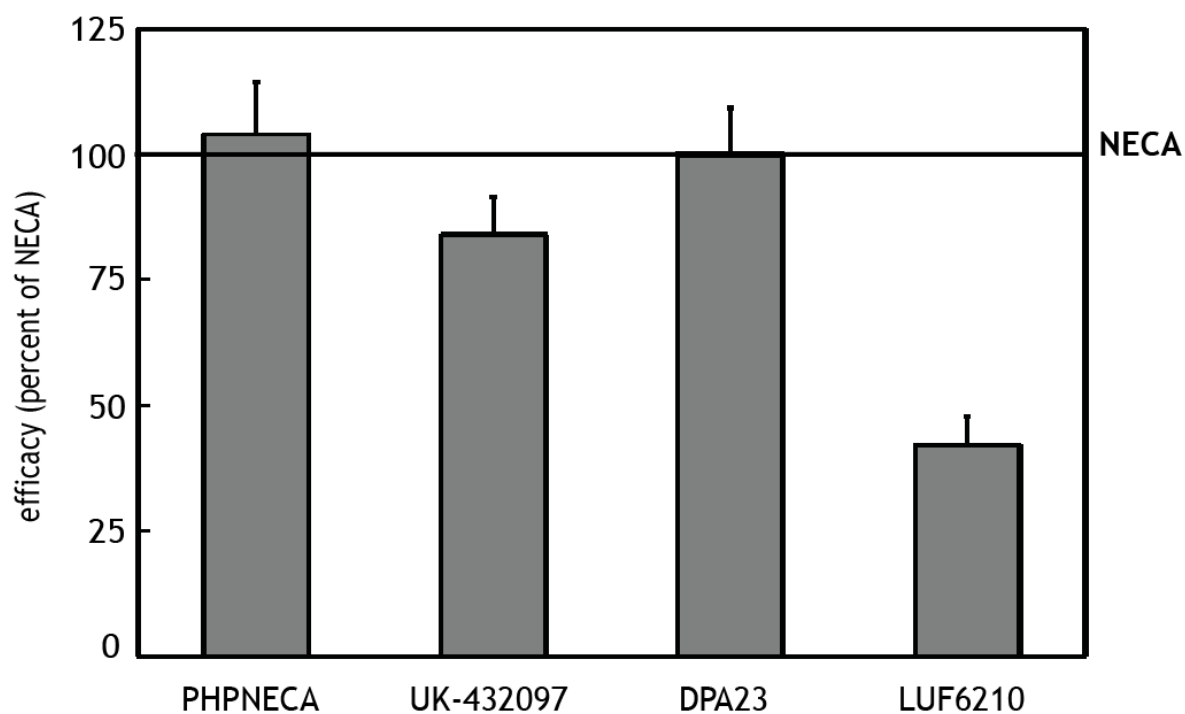
Figure 51: Efficacy of agonists for A$_{2B}$AR-mediated AC signaling. Data are expressed as percentage of the response obtained with 10 µM NECA measured in the same experiment.

We next examined the effects of the set of agonists on Ca^{2+} mobilization. Except for the nonnucleoside adenosine receptor agonist LUF6210, all tested compounds triggered a Ca^{2+}response in a concentration dependent manner (Fig. 52).
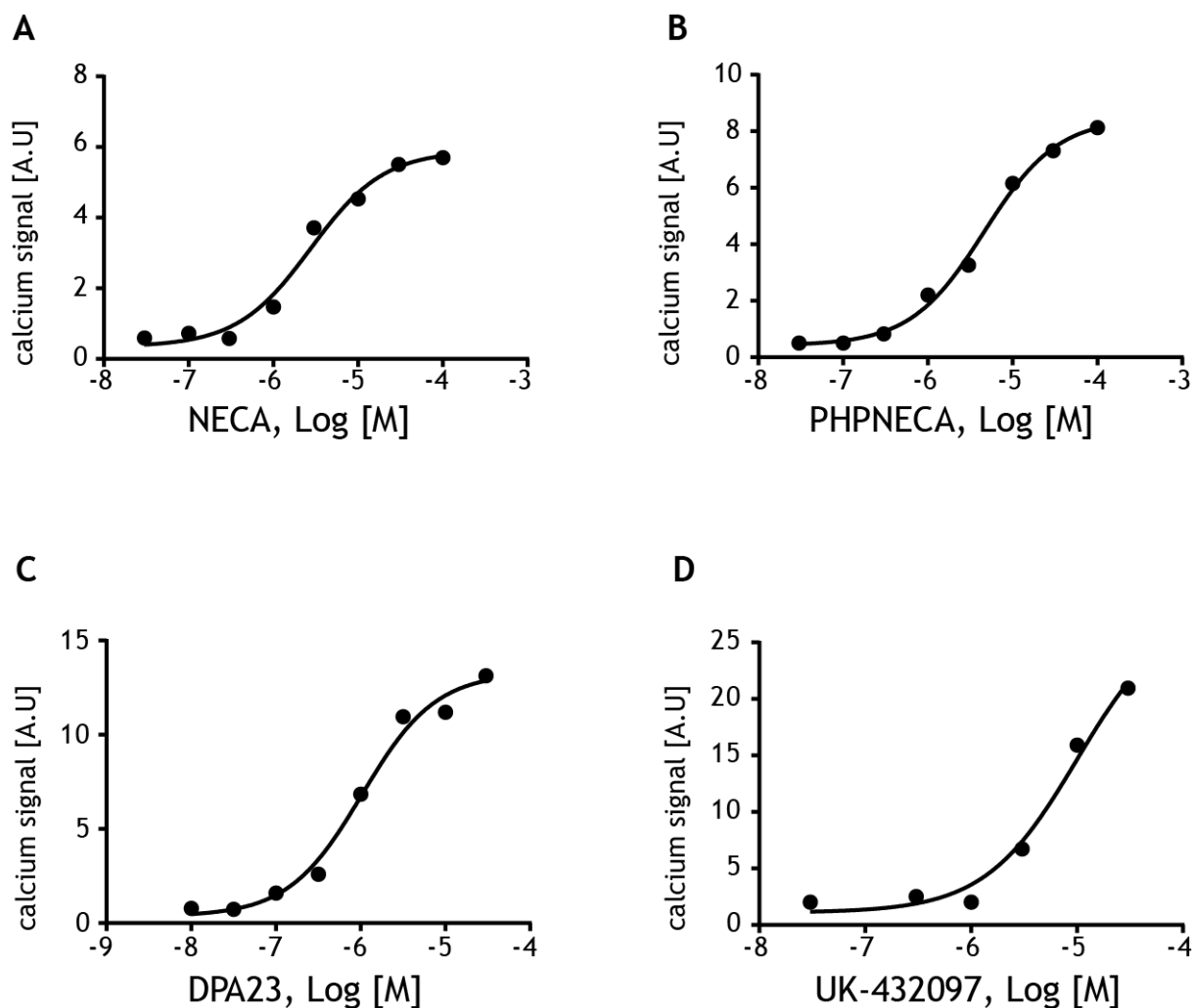
Figure 52: **Concentration-response curves for agonist-mediated Ca^{2+} release in MDA-MB-231 cells suspension.** The curves show the result of a representative concentration-response for Ca^{2+} mobilization by different AR agonists, giving EC$_{50}$ values of 2,751 (1,424-5315) (A), 4,621 (3,227-6,617) (B), 1,039 (575-1,878 (C), 9,973 (2,767-35,950) in nM with 95% confidence intervals in parentheses. Data represent response in individual experiments.

The order of potency is similar to that from the AC-assay, with DPA23 being the most potent and UK-432097 the least potent compound (Table 5) . While DPA23 showed similar efficacy to NECA, the efficacy of PHPNECA and UK-432097 was higher (Fig. 53).

Table 5: Agonist potenty for intracellular Ca^{2+} release via A$_{2B}$ARs in MDA-MB-231 cells suspension (EC$_{50}$ values, respectively, in nM with 95% confidence intervals in parentheses).

Compound	Ca^{2+}-signal, EC$_{50}$ in nM,(95% confidence intervals)
NECA	2,751 (1,424-5315)
PHPNECA	4,621 (3,227-6,617)
DPA23	1,039 (575-1,878)
UK-432097	9,973 (2,767-35,950)
LUF6210	N.D.
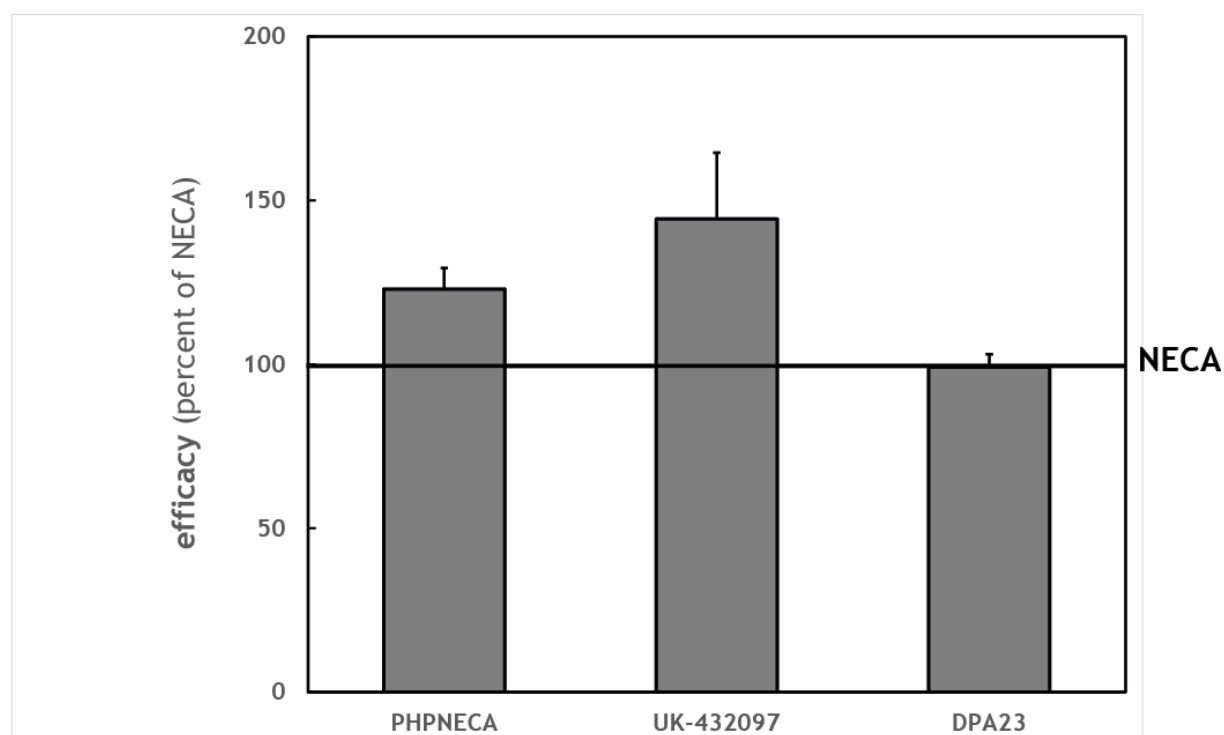
Figure 53:   Efficacy of agonists for A$_{2B}$AR-mediated Ca^{2+} signaling. The Ca^{2+}-signal is expressed as a percentage of the response obtained with 10 µM NECA measured in the same experiment.

Due to the limited solubility of UK-432097, a maximal effect could not be determined

reliably, and the concentration-response curve and the efficacy need to be interpreted carefully. The effect at 30 µM was therefore shown as the maximal effect. Taken together, all tested agonists showed agonist activity. DPA23 and UK-432097 showed EC_{50} values in the same order of magnitude for AC-activation and stimulation of Ca^{2+}-release, while NECA and PHPNECA were 5-fold less potent in the Ca^{2+}-assay. The rank order of efficacy was not maintained across the two pathways.

In addition to these pathways, the $A_{2B}AR$ has been shown to activate MAPK signaling. We observed that stimulation of the $A_{2B}AR$ in MDA-MB-231 cells mediated a reduction of ERK1/2 phosphorylation. Figure 54 shows that the diverse agonists induced the reduction of ERK1/2 phosphorylation in a concentration-dependent-manner.
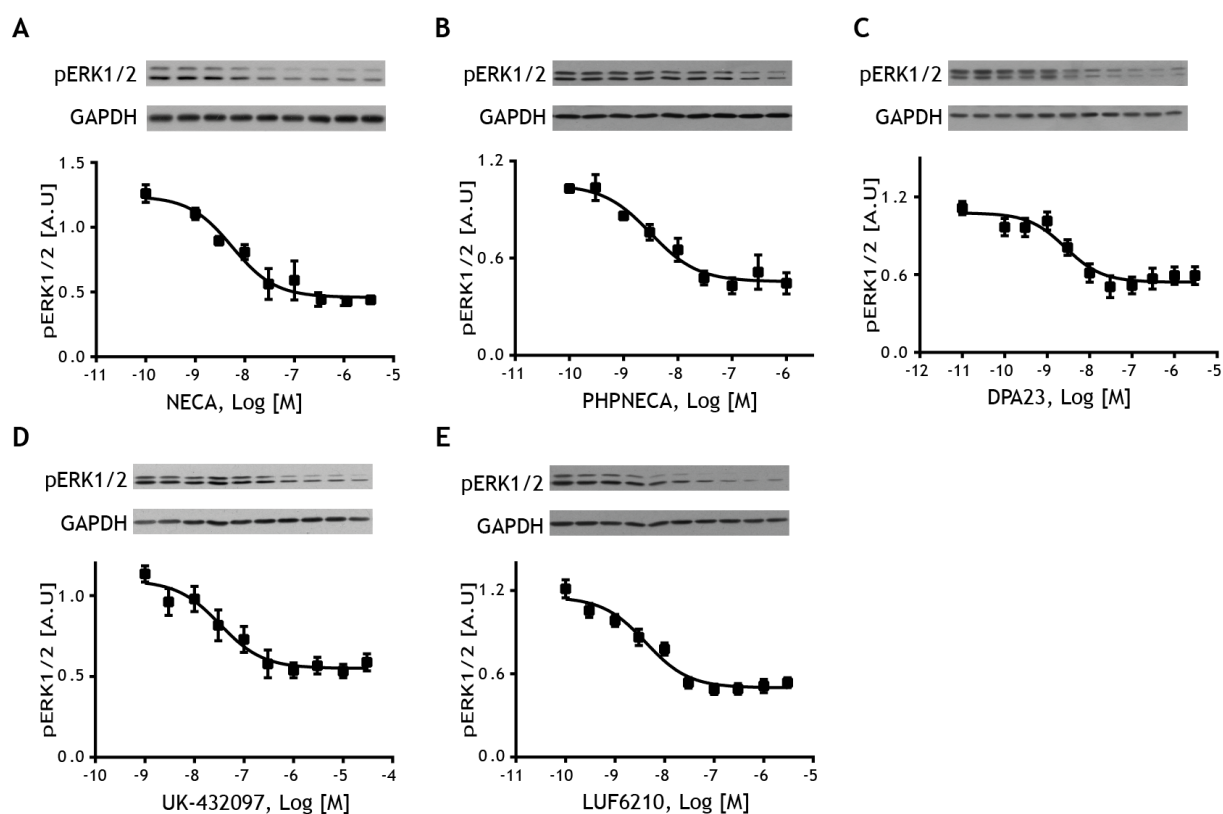
Figure 54: Concentration-response curve for agonist-stimulated pERK1/2 reduction in MDA-MB-231 cells. The Western blots are typical immunoblots representing pERK1/2 (top) and GAPDH (bottom). The curves show concentration-response curve for agonist-induced inhibition of ERK1/2 phosphorylation. Each point is the mean ± S.E. of pooled data from at least three independent experiments. The IC_{50} were 5.55 (2.54-1.21) (A), 3.13 (1.37-7.18) (B), 2.69 (1.04-6.94) (C), 3.29 (1.22-8.82) (D), 4.16 (2.33-7.40) in nM with 95% confidence intervals in parentheses.

The IC_{50} values of the various agonists are listed in Table 6. The agonists were 50-1000 times more potent in reduction of ERK1/2 than in AC signaling and Ca^{2+} response. The structurally diverse agonists were equipotent in the reduction of ERK1/2 phosphorylation. The nonselective agonist LUF6210 was the most efficacious (80%), followed by DPA23, UK-432097 and PHPNECA displaying maximum effect of 63%, 64% and 60%, respectively (Fig. 55).

Table 6: Agonist potency for pERK1/2 reduction by $A_{2B}AR$ in MDA-MB-231 whole cell lysates (IC_{50} values, respectively, in nM; with 95% confidence intervals in parentheses)

Compound	pERK1/2 (IC_{50} in nM),(95% confidence intervals)
NECA	5.55 (2.54-12.1)
PHPNECA	3.13 (1.37-7.18)
DPA23	2.69 (1.04-6.94)
UK-432097	3.29 (1.22-8.82)
LUF6210	4.16 (2.33-7.40)
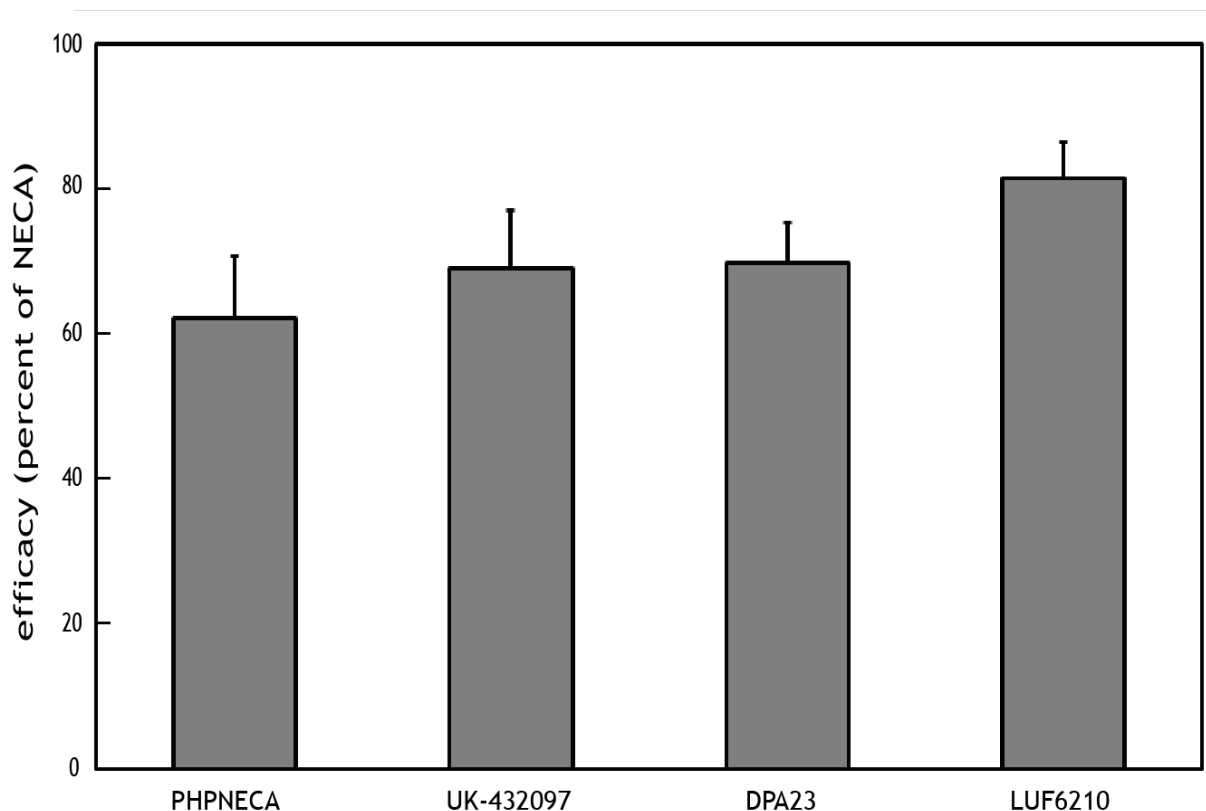
Figure 55: Efficacy of agonists for A$_{2B}$AR-mediated ERK1/2 signaling. The reduction of pERK is expressed as a percentage of the response obtained with 100 nM NECA measured in the same experiment.

Overall, the structurally diverse agonists produced a concentration-dependent increase in cAMP production and a reduction of ERK1/2 phosphorylation in MDA-MB-231 cells. LUF6210, the most potent and selective A$_{2B}$AR agonist known to date, was a partial agonist in the AC-assay, the most efficacious in reduction of ERK1/2 phosphorylation (80%), whereas it barely induced Ca^{2+} mobilization. All agonists had much higher potency for the ERK1/2 than for Ca^{2+} or cAMP responses in MDA-MB-231 cells. All things considered, no striking difference in the pharmacological profile was observed accross the set of agonists in the different assays.

In addition, we also analyzed the effects of the nonselective antagonist EFA [ANR 152], the A$_1$/A$_{2B}$ antagonist DPCPX, and ZM241385, which shows high affinity for A$_{2A}$AR, but also reasonable affinity for the A$_{2B}$AR. For each antagonist we use increasing concentrations of antagonists to reverse the effect of a single concentration of NECA (5 µM, 100 nM, 10 µM) respectively in AC, Ca^{2+} and MAPK (ERK1/2)-assays. Typical examples are illustrated, and the K$_i$ values are summarized

in Table 7. As mentioned above, stimulation of MDA-MB-231 cells with NECA resulted in an increase in intracellular cAMP, calcium mobilization, and a reduction of ERK1/2 phosphorylation. These effects were fully inhibited by pretreatment with increasing antagonist-concentrations. For each of the functional assays, there were no marked differences in potency between the tested antagonists. All antagonists had much higher potency for the Ca^{2+} than for the AC and ERK1/2 responses in MDA-MB-231 cells. The potency at $A_{2B}AR$ for the diverse antagonists determined by measuring the AC activity were similar to those in MAPK-(ERK1/2) assay, while the potency in the Ca^{2+}-assay were 20 to 30-fold higher than in the AC-assay.
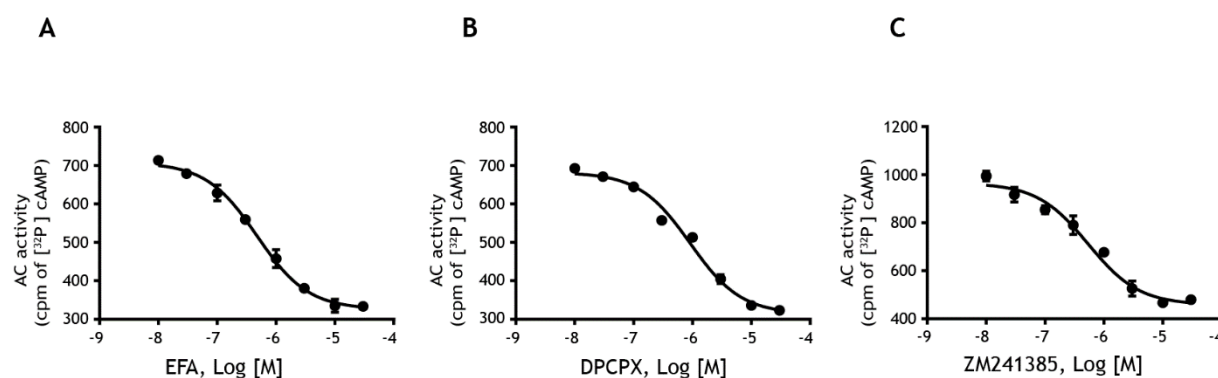
Figure 56: **Antagonism of NECA-stimulated AC response in MDA-MB-231 cells.** The curves show the result of a representative experiment (mean values of determinations in duplicate) performed at least three times. Increasing concentration of EFA (A), DPCPX (B) and ZM241385 (C) are able to reduce NECA-mediated cAMP production in MDA-MB-231 cells. The resulting K_i values are 475 (352-643) (A), 914 (648-1289) (B), 540 (316-921) (C) in nM with 95% confidence intervals in parentheses.
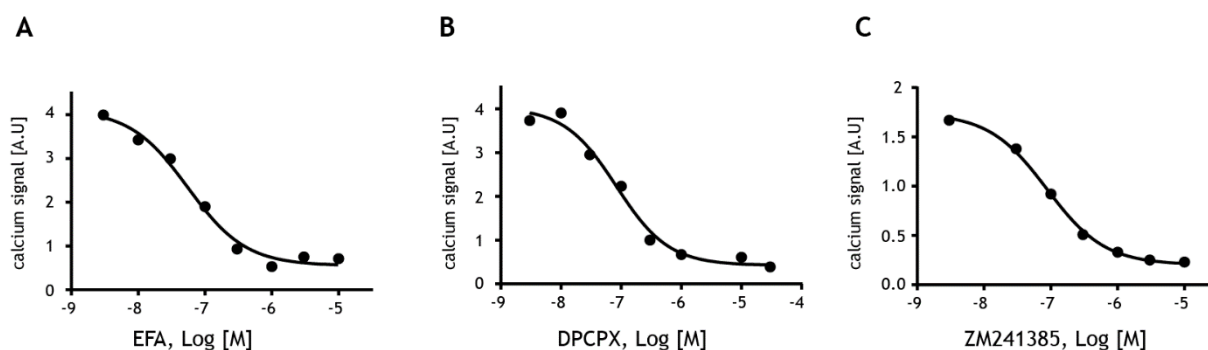
Figure 57: **Antagonism of NECA-mediated intracellular Ca^{2+} mobilization by AR antagonists in MDA-MB-231 cells.** The curves show the result of a representative experiment, performed at least three times. Increasing concentration of EFA (A), DPCPX (B) and ZM241385 (C) block NECA-mediated Ca^{2+} mobilization in MDA-MB-231 cells in a concentration-dependent manner with K$_i$ values of 55.4 (30.7-100) (A), 84.4 (44.8-155.5) (B), 87.4 (69.5-110) (C) in nM with 95% confidence intervals in parentheses.
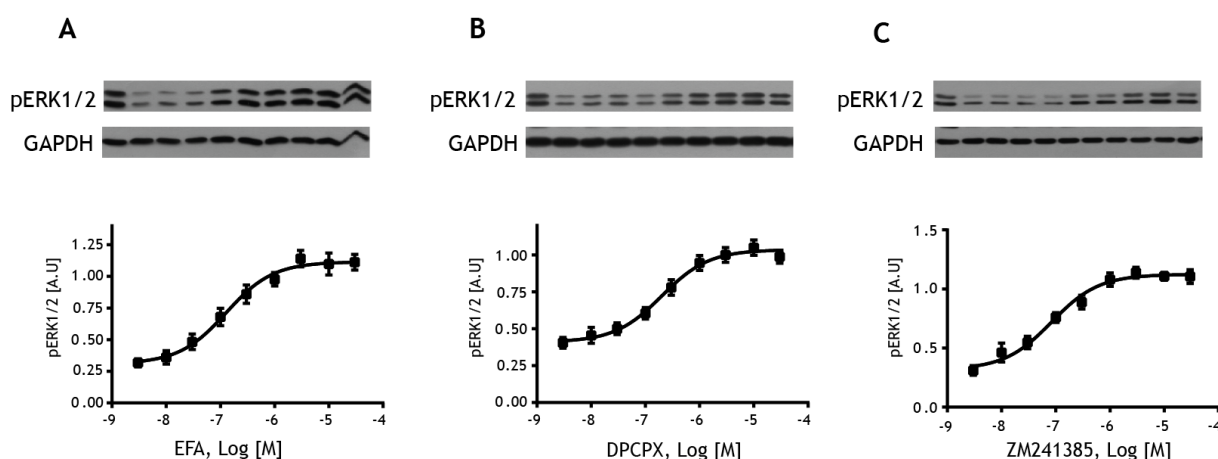
Figure 58: **Antagonism of NECA-stimulated pERK1/2 reduction by AR antagonists.** The MDA-MB-231 cells were incubated for 30 min with increasing concentrations of antagonists before stimulation with 100 nM NECA for 30 min. Representative Western blots show the concentration-dependent increase of ERK1/2 phosphorylation with increasing concentrations of the diverse antagonists (top panel) and GAPDH as loading control (bottom panel). Data are expressed relative to the basal ERK1/2 phosphorylation obtained in non-stimulated cells. Each point represents the mean ± SEM from at least three separate experiment giving K$_i$ of 127 (68.4-236) (A), 203 (114-360) (B), 84.1 (50.6-140) (C) in nM with 95% confidence intervals in parentheses.

Table 7: K_i of the diverse AR antagonists at $A_{2B}AR$ in MDA-MB-231 cells (K_i values, in nM; with 95% confidence intervals in parentheses)

Effects of antagonists on AC-activity, Ca^{2+}-mobilization and pERK1/2-reduction via $A_{2B}AR$ in MDA-MB-231 cells			
Compound	AC (Ki in nM) (95% confidence intervals)	Ca^{2+} (Ki in nM),(95% confidence intervals)	pERK1/2 (Ki in nM),(95% confidence intervals)
EFA	475 (352-643)	55.4 (30.7-100)	127 (68.4-236)
DPCPX	914 (648-1289)	84.4 (44.8-155.5)	203 (114-360)
ZM241385	540 (316-921)	87.4 (69.5-110)	84.1 (50.6-140)

Table 8: IC_{50} of the diverse AR antagonists at $A_{2B}AR$ in MDA-MB-231 cells (IC_{50} values, in nM; with 95% confidence intervals in parentheses)

Effects of antagonists on AC-activity, Ca^{2+}-mobilization and pERK1/2-reduction via $A_{2B}AR$ in MDA-MB-231 cells			
Compound	AC (IC_{50} in µM) (95% confidence intervals)	Ca^{2+} (IC_{50} in µM),(95% confidence intervals)	pERK1/2 (IC_{50} in µM),(95% confidence intervals)
EFA	6 (4-8)	0.3 (0.1-0.5)	2 (1-5)
DPCPX	12 (8-16)	0.4 (0.2-0.7)	4 (2-7)
ZM241385	7 (4-12)	0.4 (0.3-0.5)	2 (1-3)

5   Discussion

Several findings reported in the literature support an important role of adenosine in tumor development (Fishman et al., 2009). Depending on which adenosine receptor subtype is activated, adenosine can act as an anti- or pro-tumoral endogenous nucleoside (Borea et al., 2016; Borea et al., 2017). Specifically, the A$_3$AR has been reported to be the main receptor promoting anti-tumoral effects, as shown in hepatocellular carcinoma (Jacobson et al., 2018; Stemmer et al., 2013). A$_3$ARs suppress A375 cells proliferation through inhibition of ERK1/2 signaling (Merighi et al., 2005). The activation of the A$_{2A}$AR has been reported to be involved in stimulation of angiogenesis as well as in the promotion of cancer cell migration (Antonioli et al., 2016; Antonioli et al., 2013a; Antonioli et al., 2013b). Regarding the A$_{2B}$ARs, activation or inhibition may influence cell function and growth, leading to the inhibition or induction of malignant diseases. On one hand, A$_{2B}$AR activation provokes a release of angiogenic factors such as VEGF and may consequently support tumor growth (Feoktistov et al., 2002). On the other hand, blockade of A$_{2B}$ARs has been shown to slow the growth of bladder and breast tumors (Cekic et al., 2012). Conversely, stimulation of the A$_{2B}$AR in MDA-MB-231 cells leads to a reduction of ERK1/2 phosphorylation (Fig. 10), followed by proliferation reduction (Fig. 11), suggesting that A$_{2B}$ARs may inhibit the growth of these breast cancer cells. The major focus of this research was to gain a better understanding of the molecular mechanisms through which the A$_{2B}$ARs reduce the ERK1/2 phosphorylation and the downstream proliferation of MDA-MB-231 cells.

5.1   A$_{2B}$AR activation mediates the reduction of ERK1/2 phosphorylation

Our results demonstrate that A$_{2B}$AR mediates the NECA-induced reduction of ERK1/2 phosphorylation. CCPA and HEMADO selective A$_1$AR and A$_3$AR receptor agonist, respectively, failed to reduce the ERK1/2 phosphorylation (Fig. 12A), suggesting that the inhibitory effect is not mediated via A$_1$AR and A$_3$AR. The fact that the A$_{2A}$AR selective antagonist SCH58261 and the A$_3$AR selective antagonist MRS1220 did not reverse the effect of NECA rules out the involvement of A$_{2A}$AR and A$_3$AR in the NECA effect (Fig. 12A). CGS21680, an agonist with high affinity for the A$_{2A}$AR but very low affinity for the A$_{2B}$AR, was only inhibitory at high concentrations (Fig. 12B), confirming the conclusion that the effects are not mediated by A$_{2A}$AR but by A$_{2B}$AR.

The hypothesis that the inhibitory effect of NECA on ERK1/2 phosphorylation is mediated by $A_{2B}AR$ is also supported by the observation that the NECA-induced reduction of ERK1/2 phosphorylation is completely abolished by the nonselective antagonist EFA and by DPCPX, an $A_1AR/A_{2B}AR$ antagonist, at a concentration high enough to block $A_{2B}AR$ (Fig. 12E-F). Thus, only the nonselective agonist NECA, but neither agonists nor antagonists which are selective for the other adenosine receptors subtypes (A_1AR, A_3AR, $A_{2A}AR$), reduced the ERK1/2 phosphorylation or reversed the inhibitory effect of NECA. These findings provide the evidence that NECA induces the reduction of ERK1/2 phosphorylation through activation of the $A_{2B}AR$ in MDA-MB-231 cells. Our results suggest that $A_{2B}ARs$ inhibit MDA-MB-231 cell growth in part by reducing the activation of the MAPK pathway, which is in good agreement with previous results from (Dubey et al., 2005). These observations provide support to the view that activation of $A_{2B}AR$ could attenuate the growth of some type of cancer cell and argue against a stimulation of proliferation resulting from the activation of $A_{2B}AR$ as discussed by (Fernandez-Gallardo et al., 2016).

5.2 The reduction of ERK1/2 phosphorylation is cAMP and Ca^{2+} dependent

In MDA-MB-231 cells, $A_{2B}ARs$ functionally couple to G_s-mediated AC activation and to PLC-dependent intracellular Ca^{2+} mobilization (Panjehpour et al., 2005). Upon NECA stimulation, $A_{2B}AR$ triggered activation of AC that lead to an increase of intracellular cAMP levels. Presumably, cAMP exerts its downstream effects through the activation of PKA (Sands and Palmer, 2008; Tasken and Aandahl, 2004) and of the exchange factor directly activated by cAMP (EPAC) (de Rooij et al., 1998; Kawasaki et al., 1998). Cyclic AMP levels are regulated by the cyclic nucleotide phosphodiesterases (PDEs), which limit intracellular cAMP concentrations by degradation of the cyclic nucleotide (Fimia and Sassone-Corsi, 2001; Sunahara et al., 1996). Furthermore, the MAPK signaling cascade has been reported to be a target of cAMP-mediated PKA activation (Dumaz and Marais, 2005a; Osinski and Schror, 2000; Sevetson et al., 1993). AC activation by forskolin has recently been shown to enhance the activity of the chemotherapeutic agent doxorubicin in TNBC cells, via a mechanism dependent on the PKA-mediated inhibition of ERK1/2 phosphorylation. Furthermore, forskolin also increased the sensitivity of MDA-MB-231 and MDA-MB-468 TNBC cells to 5-

fluorouracil and taxol (Illiano et al., 2018), and sustain the evidence of anticancer activity mediated by cAMP/PKA-mediated ERK1/2 inhibition. Similar to these studies, a reduced amount of pERK1/2 was also observed after stimulation of AC with FSK or application of cAMP-AM and of phosphodiesterase-4 inhibitor Ro 20-1724 (Fig. 16). While EPAC activation does not appear to play any role in the reduction of ERK1/2 phosphorylation (Fig. 16), the PKA inhibitor H89 almost abolished the reduction of ERK1/2 phosphorylation without affecting the basal phosphorylation of ERK1/2 (Fig. 14A), supporting a role of the AC-cAMP-PKA-pathway in $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation. However, we observed in this study differential effects of two PKA inhibitors. The reduction of ERK1/2 phosphorylation was completely blocked by the PKA inhibitor H89 but was insensitive to KT 5720 (Fig. 14). PKA has been reported to phosphorylate CREB on Ser133, for this reason we examined the effects of these two kinase inhibitors on CREB phosphorylation in MDA-MB-231 cells. Also, their effects on NECA mediated ERK1/2 phosphorylation in HEK293 cells were investigated. In both cases, similar results as above were obtained. Our results indicate that KT 5720 is not an effective PKA inhibitor (Fig. 15). However, we could not exclude the possibility of H89 acting as a nonselective PKA inhibitor. Indeed, examination of different kinase inhibitors suggested that many of them may be quite nonspecific (Davies et al., 2000). Furthermore, the PKA inhibitor H89 has been reported to interact with the β_2-adrenergic receptor (Penn et al., 1999). The results presented above indicate that a cAMP-PKA signaling pathway is involved in the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation, but PKA-independent mechanisms could not be ruled out completely. On the other hand, we show that a rapid elevation of intracellular Ca^{2+} concentration is a key mediator in $A_{2B}AR$-induced reduction of ERK1/2 phosphorylation. PLC inhibition or chelation of intracellular Ca^{2+} profoundly reverse the reduction of ERK1/2 phosphorylation induced by $A_{2B}AR$ (Fig. 17A;18A). However, intracellular Ca^{2+} release reduced the ERK1/2 phosphorylation to a lesser extent compared to NECA (Fig. 29). AC activation and intracellular Ca^{2+} release probably work synergistically to reduce ERK1/2 phosphorylation. It is suggested that a signal cross-talk exists in MDA-MB-231 cells, whereby PKA activation and Ca^{2+} mobilization could influence one another in a positive fashion. Ca^{2+}-dependent activation of the cAMP-PKA signaling cascade has been implicated in cell survival. The biochemical link between Ca^{2+} and the activation of the cAMP-PKA pathway is primarily mediated through ACs. However, Ca^{2+} release does not play a role in

increasing cAMP levels in this study. UTP failed to modulate AC activity or to affect the potency of FSK-mediated AC response (Fig. 28), thus ruling out the existence of a calcium-dependent activation of the AC-cAMP-PKA cascade in MDA-MB-231 cells. Thus, cAMP accumulation and PLC activation followed by PKA activation and calcium mobilization, respectively, work parallel to mediate the reduction of ERK1/2 phosphorylation. However, the downstream signaling for both pathways remains unclear und needs further investigation.

5.3 The reduction of ERK1/2 phosphorylation involves *de novo* protein synthesis

The fact that the reduction of the phosphorylation of ERK1/2 is reversible in the continued presence of NECA indicates that negative regulators provide an important mechanism for MAPK (ERK1/2) control in MDA-MB-231 cells. The ability of CHX (Fig. 19), a protein synthesis inhibitor to reverse the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation, could be explained by the failure to synthesize a protein essential for the reduction of ERK1/2 phosphorylation or required to switch off the ERK1/2 activity. To the best of our knowledge, MAP kinase phosphatases (MKPs) participate in the negative feedback control of MAP kinase activation (Caunt and Keyse, 2013; Owens and Keyse, 2007). MKP-1 is encoded by an immediate-early gene, and diverse studies have shown its regulation at the transcriptional level by PKA and calcium signaling. This may indeed be the case in MDA-MB-231 cells. We observed here a profound increase in MKP-1 protein levels maximal within 30 min after $A_{2B}AR$ activation, AC activation (Fig. 20A-B), and increasing intracellular Ca^{2+} release (Fig. 21A). Our data indicate that MKP-1 expression might be the CHX-sensitive, cAMP- and Ca^{2+}-dependent event required for the reduction of ERK1/2 phosphorylation.

5.4 A$_{2B}$AR activation stimulates MKP-1 expression in a CRE dependent manner

One could only speculate on the mechanisms that regulate MKP expression in MDA-MB-231 cells. The cAMP-response element binding protein (CREB) has been shown to mediate CRE-dependent transcription (De Cesare et al., 1999). Studies reported that phosphorylation of CREB on S133 by diverse protein kinases, including PKA, is important for CRE-dependent transcription. On the other hand, CREB integrates both cAMP and elevated Ca^{2+} signals (Dash et al., 1991; Gonzalez et al., 1991; Gonzalez and Montminy, 1989; Sheng et al., 1990; Sheng et al., 1991). The human MKP-1 gene is flanked by two CREs and cAMP was found to strongly induce MKP-1 mRNA expression in NIH3T3 cells (Kwak et al., 1994). Furthermore, a calcium-sensitive block of elongation was identified within the first exon of the rat MKP-1 gene as an important element for transcription regulation of MKP-1, and TRH was shown to strongly stimulate MKP-1 transcription by enhancing initiation and elongation by Ca^{2+}-sensitive mechanisms (Ryser et al., 2001). In line with these studies, stimulation of MDA-MB-231 cells with NECA, forskolin, or UTP increase the phosphorylation of CREB at S133 (Fig. 30). It is tempting to speculate that once phosphorylated, CREB binds to CRE in MKP-genes and turns to mediate the expression of MKP-1 proteins in response to AC activation and Ca^{2+} mobilization by A$_{2B}$AR activation. In addition, the increase of intracellular Ca^{2+} may depress the calcium-sensitive block of elongation in the MKPs genes. CREB may be a key regulator in the A$_{2B}$AR-mediated MKP-1 protein increase. Elevation of cAMP and release of Ca^{2+} probably mediate the CRE-dependent transcription of the MKP-1 gene via CREB phosphorylation at S133, which in turn reduces the phosphorylation of ERK1/2 in MDA-MB-231 cells.

5.5 A$_{2B}$AR activation reduces the ERK1/2 phosphorylation through additional phosphatases

Several observations led to the notion that other phosphatases in addition to MKP-1 might play a role in the control of ERK1/2 phosphorylation. First, the increase of MKP-1 expression is slightly delayed in comparison to the reduction of the ERK1/2 phosphorylation. Second, MKP-1 knockdown was not sufficient to reverse the reduction of ERK1/2 phosphorylation. It is possible that A$_{2B}$AR activation may use other members of the MKP family or other phosphatases to induce the reduction of ERK1/2 phosphorylation seen in the MDA-MB-231 cells. An increase of MKP-2 was observed in Western blots incubated with the MKP-1 antibody (M-18) (Fig. 24B), as well as with MKP-2 antibody (Fig. 24A). The MKP-1 antibody (M-18) was generated by immunizing rabbits with a non-phosphorylated peptide corresponding to the C-terminus of MKP-1 which is partially conserved in MKP-2 (Crowell et al., 2014). This explains why the MKP-1 antibody (M-18) labels also MKP-2 and therefore showed not only a transient increase of MKP-1 but also of MKP-2, but with different kinetics. However, neither a separate nor a simultaneous knockdown of MKP-1 and MKP-2 proteins reverse the A$_{2B}$AR-mediated reduction of ERK1/2 phosphorylation (Fig. 25). As siRNA could not completely eliminate MKP-1 protein in MDA-MB-231 cells, the results obtained with this approach may not clearly reflect the role of MKP-1 in the reduction of ERK1/2 phosphorylation. Therefore, we might need MKP-knockout MDA-MB-231 cells to examine the role of MKP proteins in the A$_{2B}$AR-induced reduction of ERK1/2 phosphorylation in more detail. Nevertheless, the simultaneous increase of MKP-1 and MKP-2 proteins expression suggests a redundant mechanism that may ensure the reduction of ERK1/2 phosphorylation after A$_{2B}$AR stimulation and explain why the levels of phosphorylated ERK1/2 were not significantly different between MKP knockdown and wild-type cells (Fig. 26). MKP-1 and MKP-2 may partially substitute for each other's activity. Since the MKPs are highly regulated on a post-translational level, it is conceivable that A$_{2B}$AR activation may affect the half-life of these phosphatases through such a modification. Phosphorylation of MKP-1 and MKP-2 has been shown to enhance their stability (Brondello et al., 1999; Crowell et al., 2014; Mori Sequeiros Garcia et al., 2015). We consider the possibility of MKP proteins to be controlled through a post-translational mechanism. In MDA-MB-231 cells, the MKP-1 and MKP-2 were phosphorylated under basal conditions and NECA treatment resulted in dephosphorylation of MKP-1 at S359 (Fig. 27). This may represent a feedback inhibitory

mechanism to restrict the duration of MKPs effects and to avoid a long-term reduction of ERK1/2 phosphorylation. Furthermore, the role of phosphatase activity was corroborated by abolishing the inhibition of $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation with sanguinarine, an MKP inhibitor, or with the protein-tyrosine phosphatase inhibitor orthovanadate (Fig. 22), providing biochemical evidence that phosphatases including MKPs and PTP negatively regulate ERK1/2 phosphorylation in MDA-MB-231 cells. The effects exerted by MKPs and PTPs are generally considered to be temporarily and spatially distinct from each other (Keyse, 2000). A 'sequential phosphatase model' was proposed, in which PTPs control MAPK activity during their initial cytosolic phase whereas, after translocation to the nucleus, termination of a sustained MAPK signal would be accomplished by MKPs (Saxena and Mustelin, 2000). It is likely that the ERK1/2 phosphorylation is regulated following the sequential model proposed by Saxena and colleagues. Therefore, a proper distinction of cytoplasmic and nuclear regulation of the ERK1/2 phosphorylation is a prerequisite for consistent analysis. It might be necessary and helpful to conduct a preparation of subcellular fractions to analyze the cellular localization of the phosphatases and the kinetics of the reduction of ERK1/2 phosphorylation in the diverse cellular compartments. The possible temporal and spatial distinct phosphatase activity and their post-translational modifications may cooperate to modulate the reduction of pERK1/2. The slower effect of $A_{2B}AR$ activation on MKP expression compared to the rapid reduction of ERK1/2 phosphorylation in MDA-MB-231 cells, and the ineffectiveness of the MKP knockdown on the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation, suggested that additional mechanisms of pERK1/2 reduction independent of MKP-1/MKP-2 expression might be triggered by $A_{2B}AR$ stimulation. Thus, our data argue against the notion that the MKPs are the only determinant factor for the reduction of ERK1/2 phosphorylation. Other proteins may contribute to the early phase of the reduction of ERK1/2 phosphorylation seen in the MDA-MB-231 cells.

5.6 c-Raf-1 activity contributes to the reduction of ERK1/2 phosphorylation

MKP protein expression seems not to be the only determining factor for the reduction of ERK1/2 phosphorylation. The time course of reduction of ERK1/2 phosphorylation and of MKP expression suggested that MKP expression may mediate mainly the late phase of the reduction of ERK1/2 phosphorylation. This will only be possible if there is another pathway involved in the reduction of the early phase of ERK1/2 phosphorylation. The decrease of ERK1/2 phosphorylation was significant already after 5 min. Such rapid inhibition of the MAP kinase cascade has been shown to be caused by inactivation of c-Raf-1 (Hafner et al., 1994; Wu et al., 1993). It is possible that c-Raf-1 modulates the early phase of the ERK1/2 phosphorylation. c-Raf-1 is one of the key upstream kinases that phosphorylate MEK1/2 and subsequently activate the ERK1/2 signaling pathway. It is activated by phosphorylation when it is recruited to the plasma membrane after binding to activated Ras protein. In particular, S338 is crucial for c-Raf-1 activity, and it has been shown to be phosphorylated as a result of c-Raf-1 recruitment to the plasma membrane. On the other hand, PKA was shown to be able to directly phosphorylate c-Raf-1 on S259. Phosphorylation of S259 disrupts the recruitment of c-Raf-1 to the plasma membrane and provokes c-Raf-1 inactivation. $A_{2B}AR$ activation resulted in a rapid increase of S259 phosphorylation concomitant with a dephosphorylation of c-Raf-1 at S338 in MDA-MB-231 cells. Thus, it would be reasonable to propose that $A_{2B}AR$-mediated PKA activation blocks the recruitment of c-Raf-1 by phosphorylation of S259 (Fig. 39) and consequently reducing the dependent S338 phosphorylation (Fig. 36). Inhibition of c-Raf-1 affects the phosphorylation of MEK1/2 (Fig. 32) and reduces the subsequent downstream phosphorylation and activation of ERK1/2. While the activation of AC regulates both the increase of S259 and the reduction of S338 phosphorylation, the intracellular Ca^{2+} mobilization affects only S259 phosphorylation (Fig. 36B; 39B; 39C). UTP-mediated c-Raf-1 phosphorylation at S259 did not affect the basal phosphorylation of MEK1/2 (Fig. 32C), but a reduced ERK1/2 phosphorylation was observed (Fig. 18B). It appears plausible that both the increase of S259- and the reduction of S338-phosphorylation are required for c-Raf-1 inactivation and the subsequent downstream reduction of ERK1/2 phosphorylation in MDA-MB-231 cells. Conceivably, increasing intracellular calcium by UTP may reduce ERK1/2 phosphorylation mainly through the increase of

MKP expression. c-Raf-1 activation is a multistep process and a fully activated c-Raf-1 also requires tyrosine phosphorylation at Tyr341. This is mediated by Src or Src-like kinases (Mason et al., 1999). In MDA-MB-231 cells, the Src inhibitor PP2 decreased the phosphorylation of c-Raf-1 at S338, suggesting that phosphorylation of S338 probably requires prior phosphorylation of Tyr341. More interesting is the reduction of ERK1/2 phosphorylation in the presence of PP2 (Fig. 58). This confirms the involvement of c-Raf-1 activity in the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation. We propose that activation of PKA will prove to be one of the important events in the reduction of ERK1/2 phosphorylation in MDA-MB-231 cells. PKA-mediated reduction of c-Raf-1 activity and increased MKP expression contribute to reduce the ERK1/2 phosphorylation in MDA-MB-231 cells. Future studies examining the differential effects of NECA on MKP-1-, c-Raf-1-, and ERK1/2-activity in MDA-MB-231 cells will likely provide new insight into relationships among these proteins, and how they regulate the proliferation in concert.
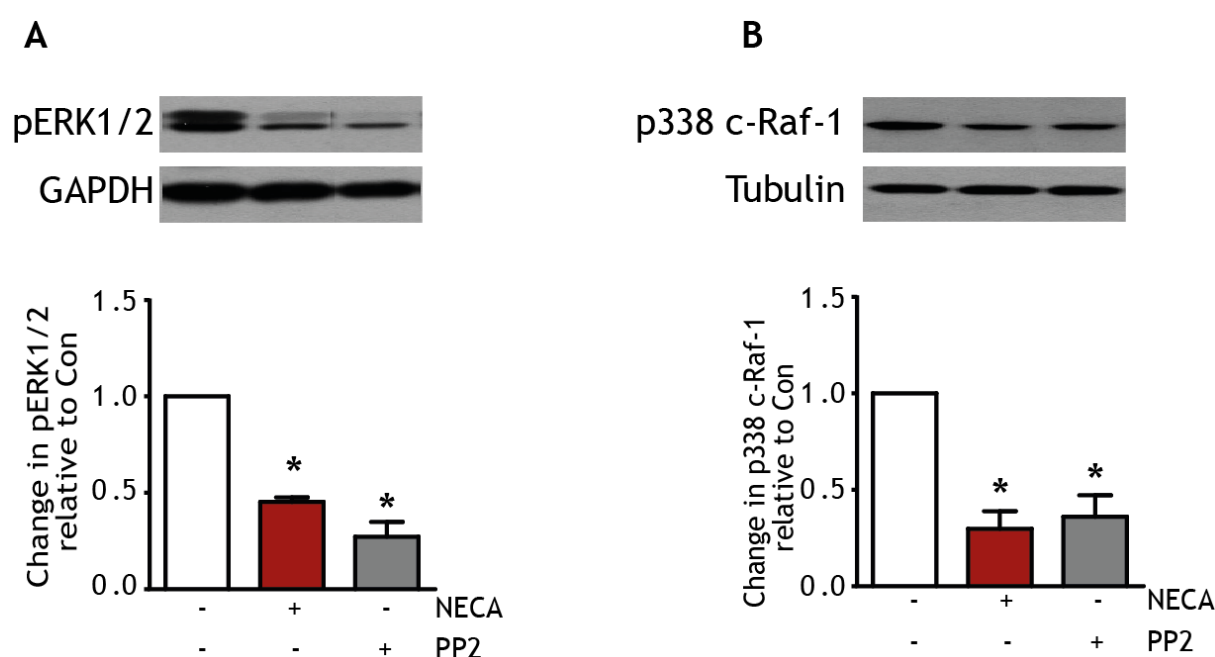
Figure 59: **Src inhibition mimics the NECA effect on pERK1/2 and p338 c-Raf-1.** MDA-MB-231 cells were treated with 100 nM NECA and 10 µM PP2 the Src inhibitor for 30 min. ERK1/2 phosphorylation and c-Raf-1 phosphorylation was examined. The Western blots show representative experiments, the columns represent mean values from n = 3 (A) and 4 (B) independent experiments, respectively (* p < 0.001 significantly different from untreated cells).

5.7   How does A$_{2B}$AR activation affect other MAPKs?

Besides ERK1/2, MAPKs include the c-Jun N-terminal kinases 1-3 (JNK1-3)/stress-activated protein kinases (SAPK1A, 1B, 1C), and the p38 isoforms (p38α, β, γ, and δ).

5.7.1   A$_{2B}$AR activation increases the phosphorylation of p38 MAPK

MAPK p38 regulates many cellular events from cell cycle progression to apoptosis. The MAPK p38 activity is controlled by intracellular signaling pathways such as the cAMP pathway. The AC activator forskolin can induce phosphorylation of the p38 MAP kinase in a PKA-dependent manner (Delghandi et al., 2005; Hansen et al., 2000). On the other hand, forskolin has been shown to inhibit p38 MAP kinase phosphorylation in endothelial cells (Rahman et al., 2004). In contrast to NECA and UTP, AC activation by forskolin did not increase the p38 phosphorylation after 5 min, but significantly promoted the phosphorylation after 1h (Fig. 44). The finding that activation of A$_{2B}$AR by forskolin failed to increase p38 MAPK phosphorylation strongly suggests that the Ca^{2+}-pathway leads to the transient p38 phosphorylation. PKA-independent activation of p38 in colon cancer cells was reported by (Nishihara et al., 2004). Also elevated intracellular Ca^{2+} was involved in p38 MAPK-induced neuronal cell death by pneumolysin (Stringaris et al., 2002). We speculate that in MDA-MB-231 cells, A$_{2B}$AR-mediated p38 phosphorylation is a Ca^{2+}-pathway dependent. A novel and unexpected MAPKK-independent mechanism of activation of p38 MAPKs has been proposed. TAB1, originally known as a binding protein and an activator of TAK1, directly binds to and induces activation of p38α. This study indicates that the enzymatic activity of p38 can be enhanced by autophosphorylation (Ge et al., 2002). In line with this study, inhibition of p38 by SB203580 blocked the NECA-mediated increase of p38 (Fig. 45), suggesting that intracellular Ca^{2+} release activates p38 phosphorylation through a mechanism dependent on p38 kinase activity. The MAPK p38 was inactivated by MKPs through dephosphorylation (Owens and Keyse, 2007). A feedback control of MKP-1 expression by p38 was described by (Hu et al., 2007). In time-course experiments we observed a correlative relationship between p38 activation and MKP expression and the concomitant p38 dephosphorylation. This suggests that p38 activity may induce the expression of the MKPs which in turn terminated the increase of p38

phosphorylation. In resting cells, inactive p38 resides primarily in the cytosol. Phosphorylated p38 translocates into the nucleus and is dephosphorylated by MKPs. Thus, the intracellular localization of p38 depends on its phosphorylation. Hence, the next key task is to investigate the nuclear and cytoplasmic regulation in order to precisely characterize the effect of $A_{2B}AR$ on p38 phosphorylation and the biological function of p38 activation in MDA-MB-231 cells. It must be mentioned that the role of p38 is cell type and stimulus-dependent. While p38 signaling was shown to promote cell death in some cells, it also enhances survival, cell growth and differentiation in other cells lines. Thus, the effects of p38 phosphorylation on MDA-MB-231 cell proliferation should be examined.

5.7.2 $A_{2B}AR$ activation reduces the phosphorylation of JNK

In addition to ERK1/2, GPCRs were also shown to activate JNK/SAPK, another member of the MAP kinase family. JNK signaling pathway has been implicated in many pathological conditions, including cancer (Davis, 2000; Kyriakis and Avruch, 2001). The JNK activity is controlled by diverse intracellular signalings pathways. Cyclic AMP has been shown to inhibit JNK activation (Hsueh and Lai, 1995; Rao and Runge, 1996). Consistent with this report, and like the $A_{2B}AR$-mediated reduction of ERK1/2 phosphorylation, we observed a reduction of JNK phosphorylation in MDA-MB-231 cells. The treatment of MDA-MB-231 cells with forskolin or other cAMP elevating agents led to reduction of JNK phosphorylation in a time-dependent manner (Fig. 40). On the other hand, intracellular calcium elevation was required for JNK activation (Matsuoka and Igisu, 1998; Yu et al., 2000). These findings suggest that activation of JNK may relate to intracellular Ca^{2+} increase. Conversely, we observed a reduction of JNK phosphorylation (Fig. 41A). Given that PKA or PLC is necessary for JNK phosphorylation (Fig. 41B-C), the question arises as to how the reduction of JNK phosphorylation is accomplished. PKA or PLC could not directly dephosphorylate JNK, thus suggesting the involvement of an intermediary substrate. We propose that similar to the reduction of ERK1/2 phosphorylation, the induction of MKPs as described before are required for the inhibition of JNK activation. Furthermore, we found that knockdown of MKP expression partially reverses the reduction of JNK phosphorylation (Fig. 43). Therefore, MKPs are indeed involved in the cAMP- and Ca^{2+}-mediated JNK reduction. Moreover, Pedram and cowokers have

reported that MEK indirectly activated JNK in VEGF-stimulated EC cells (Pedram et al., 1998). It is possible that the MEK pathway may contribute to the reduction of JNK phosphorylation in MDA-MB-231 cells. As shown for ERK1/2, JNK and p38 are also implicated in various cellular processes including transcription. A study in colorectal cancer cells suggested that the ERK MAPK pathway, but not the JNK pathway or the p38 MAPK pathway, is a major regulator of cell proliferation. These MAPKs were shown to act cooperatively in various biological functions. For example, TFs are targeted by one, or frequently by two and even three of the MAPK pathways. The TF Elk1 is regulated by ERK1/2, JNKs and p38s (Yang et al., 1998) and c-Jun is regulated by JNKs and ERK1/2 (Morton et al., 2003). Furthermore, MAPKs activation and in particular the balancing between pERK1/2 and p-p38 has been shown to play a key role in the establishment of tumor dormancy in certain models (Aguirre-Ghiso, 2007). We have observed that $A_{2B}AR$ activation produces an inhibitory effect on ERK1/2- and JNK-phosphorylation, while a transient increase of p38 phosphorylation was noticed. Furthermore, a reduction of cell proliferation was observed. Whether, the diverse MAPKs cooperate in MDA-MB-231 cells to regulate the cell proliferation merits further research. Because ERK1/2, JNK, and p38 are capable of inducing either apoptosis or cell proliferation, it will be important to assess the role of ERK1/2, JNK, and p38 pathways in MDA-MB-231 cells following $A_{2B}AR$ activation by using selective MAPK-inhibitors.

5.8 Functional selectivity of $A_{2B}AR$ ligands

Stimulation of the G_s-coupled $A_{2B}AR$ mediates an activation of AC and in addition triggers a Ca^{2+} signal. In the estrogen-receptor negative cancer cell line MDA-MB-231, which is expressing $A_{2B}ARs$ as the sole AR subtype, reduction of ERK1/2 phosphorylation was found as an additional $A_{2B}AR$ signal. The MAP-kinase signaling is associated with the control of growth, proliferation and differentiation of cells and as such might serve as a promising target for tumor treatment, while Ca^{2+} signal has long been known to play a crucial role in angiogenesis. Thus, the identification of $A_{2B}AR$-ligands (or other ligands for G_s-coupled receptors) with bias toward the ERK1/2 pathway would have a considerable therapeutic potential, as such ligands will provide the opportunity to selectively stimulate a desired activity like reduction of ERK1/2 phosphorylation. This would avoid stimulation of pathways that are

responsible for side effects, like in the case of MDA-MB-231 cells the release of intracellular Ca^{2+}, an important event in angiogenesis (Kohn et al., 1995). Besides diverse adenosine derivatives we studied the functional selectivity of LUF6210, a nonnucleoside adenosine receptor agonist. LUF6210 is a partial agonist with 300-fold selectivity for $A_{2B}AR$ over other AR subtypes (van der Hoeven et al., 2011). As can be seen in table 3, LUF6210 showed significantly higher affinity than NECA in membrane binding studies in CHO cell lines stably expressing $A_{2B}ARs$. In MDA-MB-231 cells, in each functional assay in which we determined EC_{50} values, no major difference was observed between the pharmacological profiles of the set of ligands in the same assay. But when comparing the different assays with each other, the reduction of ERK1/2 phosphorylation occurred with 50-400 times higher potency than the stimulation of AC activity and the Ca^{2+} signal. The activation of AC-PKA- and PLC-Ca^{2+}-pathways was found to synergically mediate the reduction of ERK1/2 phosphorylation, thereby increasing the potency to promote the reduction of ERK1/2 phosphorylation. Furthermore, ERK1/2 is a pathway with significant amplification. The reduction of ERK1/2 phosphorylation is mediated through enzymatic signal transduction cascades. Signal amplification may occur at the level of each enzyme involved sequentially in the signaling pathway, e.g., c-Raf-1, MEK1/2 and MKPs. This could explain why the EC_{50} for all tested agonist is much lower than the concentration needed to cause half maximal receptor occupancy. LUF6210, which has a nonnucleoside scaffold, did not provoke Ca^{2+} mobilization, thus supporting the suggestion that structurally distinct compounds have the potential to stimulate responses that differentiate from that of the endogenous agonist. Hinz et al., (2014) observed in a Ca^{2+} assay that LUF6210 was clearly more efficacious in recombinant HEK293 cells overexpressing human $A_{2B}ARs$ than in non-transfected HEK293 cells, but significantly less efficacious than NECA and adenosine. This suggests that LUF6210-mediated Ca^{2+} response may be limited by the concentration of receptors, or the absence of amplification in the Ca^{2+} signaling cascade in MDA-MB-231 cells. It was reported, that partial agonists may not bind long enough to the receptor to induce a maximal response (Hoeren et al., 2008). We speculate that LUF6210 might stabilize a very different receptor conformation than the full agonist NECA, a conformation which blocks the mobilization of Ca^{2+}. Accordingly, it would be of interest to interrogate the possibility of antagonistic behavior of the non-adenosine derivative LUF6210 on Ca^{2+} response in particular. It is worth noting that by using the

CLARIOstar plate reader (BMG), we observed that LUF6210 excitation and emission overlap with FURA-AM and may prevent the monitoring of a Ca^{2+} response. Whether LUF6210 fluorescence could be accounted for by contamination must be proven. Moreover, this study demonstrates that the least potent agonist UK-432097 had the highest efficacy for the Ca^{2+} response while being as efficacious as NECA in cAMP accumulation and in the reduction of ERK1/2 phosphorylation. As it is difficult to establish a full calcium-concentration-response curves with UK-432097 mainly due to its low affinity and insolubility at high concentration, its efficacy could not be determined precisely. Looking at the efficacies of the rest of agonists, we found that NECA and DPA 23 were both highly efficacious in all assays. In comparison to NECA, while showing similar or slightly higher efficacy respectively in AC and Ca^{2+} assay, PHPNECA is the least efficacious agonist in reduction of ERK1/2 phosphorylation. To further investigate the pharmacological properties of the $A_{2B}AR$ in MDA-MB-231 cells, also antagonist studies were performed. An antagonist is devoid of intrinsic activity. It acts by preventing or altering the binding of an agonist. In MDA-MB-231 cells, regardless of the assay, similar potencies were obtained for the tested antagonists. But when comparing the different assays with each other, while higher potencies were observed in Ca^{2+} assay, the potencies observed in AC- and ERK1/2-pathway were in the same order of magnitude (table 8). The diverse antagonists antagonized the Ca^{2+} signal with 20-30 times higher potency than the AC activity and 5-10 times higher than the reduction of ERK1/2 phosphorylation. The potency of the diverse antagonists in AC and in ERK1/2 response mirrors the affinity of the antagonists for the $A_{2B}AR$. For example, while DPCPX potency toward inhibition of NECA-mediated AC activation (IC_{50} = 12 μM) and reduction of ERK1/2 phosphorylation (IC_{50} = 4 μM) are in line with the IC_{50} = 3.15 μM obtained for the AC activity by (Klotz et al., 1998), its potency to reverse the Ca^{2+} mobilization is much higher (IC_{50} = 0.4 μM). We obtained discrepant results, with increasing potency of the antagonists in the Ca^{2+} response. We do not have any explanation for this phenomenon, which have been observed between AC activity in membrane preparations and accumaltion of cAMP in whole cells (Klotz et al., 1998). It is noteworthy to mention, that Ca^{2+} assay was performed in whole cells, wheras AC- and ERK1/2-assay are performed in membrane and in whole cell lysates respectively.

5.9   The β-AR as another G$_s$-coupled receptors in MDA-MB-231 cells

The activation of an endogenous β-AR in several breast cancer cell lines is associated with a PKA-dependent decrease of cell proliferation. Previous work showed that the β-AR agonist ISO suppresses the growth of human MDA-MB-231 breast cancer cells through increased cAMP production, and this effect is blocked by propranolol and mimicked by 8-BrcAMP (Slotkin et al., 2000). Most likely, ISO mediates a reduction of ERK1/2 phosphorylation in MDA-MB-231 cells (Fig. 46A). The observed reduction in ERK1/2 phosphorylation can certainly lead to a reduction of MDA-MB-231 cells proliferation. In addition, we have found that triggering cells with ISO raises intracellular levels of Ca^{2+} (Fig. 46C). We showed by various pharmacological approaches in MDA-MB-231 cells, that β-AR is stimulating the same pathway used by A$_{2B}$AR to reduce the phosphorylation of ERK1/2 (Fig. 47; 48). Thus, the reduction of ERK1/2 phosphorylation could be assigned to two independent G$_s$-coupled receptors in MDA-MB-231 cells. Taken together, activation of A$_{2B}$AR or of a G$_s$-coupled receptor like β-AR leads to AC activation followed by the production of cAMP and the activation of PKA, and on the other hand to an increase in the intracellular Ca^{2+} release. Consequently, AC-activation and Ca^{2+}-mobilization induce the reduction of ERK1/2- and JNK-phosphorylation. In contrast, our studies showed that the A$_{2B}$AR-mediated p38-phosphorylation requires p38 MAPK catalytic activity, suggesting that autophosphorylation is involved. Interestingly, the activation kinetics were the same when we compared the reduction of ERK1/2 phosphorylation after NECA, FSK, ISO, and UTP application. Futhermore, A$_{2B}$AR-mediated cAMP production and Ca^{2+} mobilization act synergistically to reduce ERK1/2 phophorylation. However, the NECA-mediated reduction was stronger when compared to UTP treated cells (Fig. 29). Thus, the Ca^{2+}-dependent mechanism appears to mediate a small portion of signals probably via increased MKPs expression. MKP may limit the temporal activity of the MAPK by reduction of ERK1/2 phosphorylation. A$_{2B}$AR and the downstream MKP might therefore represent interesting targets for the inhibition of proliferation of fast-growing cancer cells. Even when inhibition of phosphatases reversed the reduction of ERK1/2 phosphorylation, our findings characterized c-Raf-1 inhibition to cooperate with MKPs to produce the maximal reduction of ERK1/2 phosphorylation. Further studies should be carried out to fully comprehend how A$_{2B}$AR or G$_s$ protein-coupled receptor activation regulate the proliferation of MDA-

MB-231 cells. These mechanisms must be established in order to fully comprehend the role of A_{2B}ARs in MDA-MB-231 cells. A proposed schema for the A_{2B}AR-mediated inhibition of ERK1/2 signaling pathway is presented in Fig. 60.
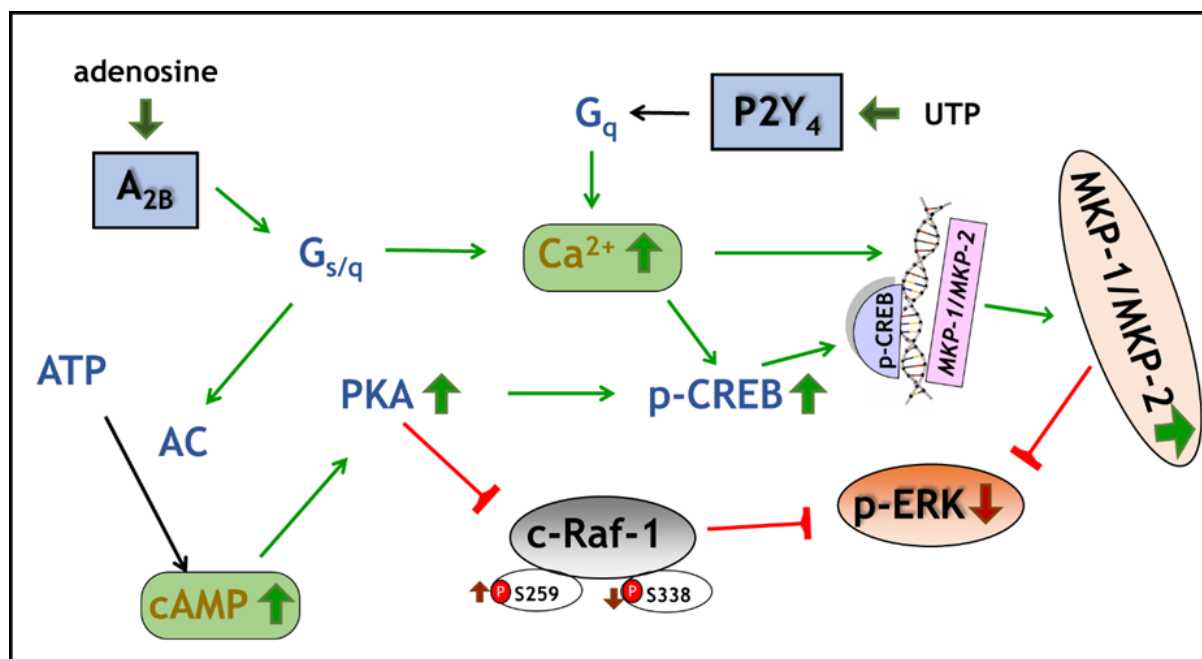
Figure 60: **Schematic representation of the reduction of the ERK1/2 phosphorylation.** A_{2B}AR engages two major parallel pathways involving c-Raf-1 and MKPs. Once cAMP is activated, it activates PKA and Ca^{2+} release. Then PKA phosphorylates and inhibits c-Raf-1. c-Raf-1 inhibits MEK1/2 which in turn reduces pERK1/2. Activated PKA and Ca^{2+} induced both CREB dependent transcription of MKP-1 and MKP-2 which turn to contribute in the reduction of pERK1/2. The increase of Ca^{2+} may depress the calcium-sensitive block of elongation in the MKPs genes. β-AR an other G_s-coupled receptor is stimulating the same pathway used by A_{2B}AR to reduce the phosphorylation of ERK1/2. The reduction of pERK1/2 could be extended to G_s-coupled receptors.

It is worth noting that A_{2B}AR effects vary depending on the cell type. On one hand, inhibition of A_{2B}AR slows the growth of MB49 bladder and 4T1 breast tumors in syngeneic mice (Cekic et al., 2012), PC3 prostate cancer cell lines (Wei et al., 2013)(Wei.et al., 2013) and colorectal cancers (Ma et al., 2010). On the other hand, activation of A_{2B}AR is linked to the inhibition of ovarian cancer proliferation (Hajiahmadi et al., 2015) and the growth of cardiac fibroblasts by inhibition of MAP kinase activity (Dubey et al., 2001). In contrast, some studies exclude A_{2B}AR from a role in the adenosine-induced cells proliferation. Despite the functional presence of A_{2B}AR in human melanoma A375 (Merighi et al., 2001), A_{2B}AR is not involved in the

adenosine-induced A375 cells proliferation (Merighi et al., 2002). Likewise, a functional form of $A_{2B}AR$ was found in the human breast cancer cell lines MCF-7 and MDA-MB-468, but its involvement in growth modulation was not seen in these cells (Panjehpour and Karami-Tehrani, 2007). The current study revealed that $A_{2B}AR$ activation reduced the proliferation of the MDA-MB-231 cells. One explanation may be that the $A_{2B}AR$ level in these cells (MCF-7, MDA-MB-468) is not sufficient for productive effector coupling. On the other hand, the conflicting effects of $A_{2B}AR$-activation could be due to the coexpression of adenosine receptor subtypes in the same cells. The presence of other AR subtypes probably reduces or removes the $A_{2B}AR$ response. Depending on cell studied, either one of these receptor subtypes can take the lead and play a dominant role in the adenosine-mediated effects. The expression of $A_{2B}AR$ as the sole AR subtype is characteristic of the MDA-MB-231 cells. For example, the expression of the transcript of all adenosine receptors was observed in the human breast cancer cell lines MCF and MDA-MB-468 (Panjehpour and Karami-Tehrani, 2007), while the HCC1806, HCC1937 breast cancer cells appeared to be devoid of any detectable amount of ARs. Because the expression of adenosine receptor subtypes varies depending on the cell type, a complete pharmacologic characterization of the number of expressed adenosine receptors is required to investigate the effect of $A_{2B}AR$ on cancer cell growth. Addressing the question of which adenosine receptors are present in cancer tissues and how these receptor subtypes affect cancer growth is crucial in order to devise potential new treatment options for cancer.

6 Summary

Recently, it was shown that MDA-MB-231 breast cancer cells express very high levels of the $A_{2B}AR$ as the sole adenosine receptor subtype, and stimulation of the $A_{2B}AR$ in MDA-MB-231 cells triggers an unusual inhibitory signal on ERK1/2 phosphorylation. The ERK1/2 pathway is reported to be associated with the control of growth, proliferation and differentiation of cells and as such might serve as a promising target for tumor treatment. The present study investigated signaling mechanisms involved in linking $A_{2B}AR$ to ERK1/2 phosphorylation in MDA-MB-231 cells. The $A_{2B}AR$ mediated reduction of ERK1/2 phosphorylation and of proliferation of MDA-MB-231 cell is in good agreement with previous results from (Dubey et al., 2005). These observations provide support to the hypothesis that activation of $A_{2B}AR$ could attenuate the growth of some types of cancer cell and argue against a stimulation of proliferation resulting from the activation of $A_{2B}AR$ as discussed by (Fernandez-Gallardo et al., 2016). AC activation by forskolin has recently been shown to enhance the activity of the chemotherapeutic agent doxorubicin in TNBC cells via a mechanism dependent on the PKA-mediated inhibition of ERK1/2 phosphorylation. Furthermore, forskolin also increased the sensitivity of MDA-MB-231 and MDA-MB-468 triple negative breast cancer cells to 5-fluorouracil and taxol (Illiano et al., 2018), and sustains the evidence of anticancer activity mediated by cAMP/PKA-mediated ERK1/2 inhibition. Similar to these studies, a reduced amount of pERK1/2 was also observed after stimulation of AC with FSK, application of cAMP-AM or inhibition of PDE-4. The inhibition of ERK1/2 phosphorylation was mimicked by UTP and abolished with the PLC inhibitor U73122 or by chelating intracellular Ca^{2+} with BAPTA-AM. These results point to an important role for both cAMP and Ca^{2+} signaling in the pathway leading to a decrease in ERK1/2 phosphorylation. This study encourages the idea that $A_{2B}AR$ could be used as target in cancer therapy. But $A_{2B}AR$ did not only stimulate signaling cascades associated with cell survival and proliferation reduction, but also key phases relevant in angiogenesis like Ca^{2+} mobilization (Kohn et al., 1995). Whereas the potency toward AC and Ca^{2+} are similar for the diverse agonists, the potency to promote ERK1/2 reduction is much higher. Interestingly, the proliferation of MDA-MB-231 cells is inhibited by low nanomolar agonist concentration which is inactive in Ca^{2+} mobilization. This means that it is certainly possible to reduce the proliferation without promoting angiogenesis. LUF6210 is

particularly interesting when considering that it preferentially stimulates a reduction in ERK1/2 phosphorylation over Ca^{2+} and therefore may not promote angiogenesis. LUF6210 is therapeutically appealing as adjuvant in treatment of cancer. Given that stimulation of AC can activate a reduction of ERK1/2 phosphorylation and proliferation in cancer cells, agonist bias toward G_s-AC-PKA-mediated ERK1/2 inhibition represent a potential therapy of various malignancies. The fact that the reduction of ERK1/2 phosphorylation followed by reduced proliferation observed in MDA-MB-231 cells were mediated by the activation of the $A_{2B}AR$ illustrates the importance of this receptor subtype in cancer. $A_{2B}ARs$ must be considered as a key factor in cancer treatment and deserve attention for the development of new therapeutic strategies.

7 Zusammenfassung

Adenosin reguliert eine Reihe physiologischer Funktionen über die vier ARs, die zur Familie der GPCR gehören. Adenosin beeinflusst das Zellwachstum sowohl positiv als auch negativ. Dabei spielen die MAPK eine wichtige Rolle. Diverse Studien haben gezeigt, dass die Aktivierung alle ARs Subtypen zur Phosphorylierung der MAPK ERK1/2 führt. Es gibt immer mehr Hinweise auf die Beteiligung des $A_{2B}AR$ am Wachstum und der Progression von Tumoren. Die MDA-MB-231 Brustkrebszellen weisen eine hohe Expressionsrate des $A_{2B}AR$ als einzige ARs Subtypen auf. Zusätzlich zu AC-Aktivierung und intrazellulärer Ca^{2+}-Freisetzung führt die Stimulation des $A_{2B}AR$ der MDA-MB-231-Brustkrebszellen zur Reduktion der ERK1/2 Phosphorylierung. NECA, der unselektive AR-Agonist, führt zu einer zeit- und konzentrationsabhängigen Inhibition der ERK1/2 Phosphorylierung. Auch eine signifikante Reduktion der Proliferation der MDA-MB-231 Brustkrebszellen wurde beobachtet. Unsere Ergebnisse deuten darauf hin, dass $A_{2B}ARs$ das Wachstum von MDA-MB-231 Zellen hemmen, indem sie die Aktivierung des ERK1/2 reduzieren, was in gutem Einklang mit den Ergebnissen von (Dubey et al., 2005) steht. Diese Ergebnisse unterstützen die Ansicht, dass die Aktivierung von $A_{2B}AR$ das Wachstum von bestimmten Arten von Krebszellen hemmt, und wiederspricht dem fördernden Effekt des Wachstums von $A_{2B}AR$ beschrieben in (Fernandez-Gallardo et al., 2016). Die AC-Aktivierung durch Forskolin erhöht den Effekt des Chemotherapeutikums Doxorubicin in TNBC Zellen. Darüber hinaus erhöhte Forskolin auch die Empfindlichkeit von MDA-MB-231 und MDA-MB-468 TNBC auf 5-Fluorouracil und Taxol (Illiano et al., 2018) und bestätigt die anti-Krebs-Aktivität von reduzierter ERK1/2 Phosphorylierung, die von cAMP/PKA abhängig ist. Ähnlich zu diesen Studien reduziert sowohl eine Behandlung der MDA-MB-231 Zellen mit Forskolin oder mit cAMP-AM, als auch Hemmung der PDE-4 die ERK1/2 Phosphorylierung. Die durch $A_{2B}AR$-vermittelte Reduktion der pERK1/2 ist in Anwesenheit des PKA Inhibitors H89 gehemmt. Die Reduktion der ERK1/2 Phosphorylierung wurde durch den PLC-Inhibitor U73122 und den Ca^{2+} Chelator BAPTA-AM gehemmt. Außerdem induziert die Ca^{2+} Freisetzung bei UTP die Reduktion der ERK1/2 Phosphorylierung. Diese Ergebnisse weisen auf eine wichtige Rolle von cAMP und Ca^{2+} in der $A_{2B}AR$-vermittelten Hemmung der ERK1/2 Phosphorylierung hin. Eine solche Abnahme kann als Folge der Hemmung einer Kinase oder Stimulation einer Phosphatase auftreten. Wir untersuchten die MKPs, ein negativer Regulator der

MAPK-Aktivität. Unsere Ergebnisse zeigen, dass die Stimulation des $A_{2B}AR$ in MDA-MB-231 Zellen zu erhöhter MKP-1 und MKP-2 Expression führt. Dieser Effekt bietet einen neuartigen Mechanismus für die $A_{2B}AR$-vermittelte Reduktion der ERK1/2 Phosphorylierung. Der $A_{2B}AR$ und die induzierten Phosphatasen MKP-1 und MKP-2 könnten daher interessant für die Hemmung der Proliferation schnell wachsender Krebszellen sein. Auch wenn die Hemmung von Phosphatasen Aktivitäten die Reduktion der ERK1/2 Phosphorylierung rückgängig macht, deuten unsere Ergebnisse auf eine Beteilung der c-Raf-1 in der Reduktion der ERK1/2 Phosphorylierung hin. Es konnte gezeigt werden, dass die Aktivierung der β-AR Rezeptoren ähnliche Signale wie $A_{2B}AR$ in MDA-MB-231 Zellen regulieren. Daher kann die Reduktion der ERK1/2 Phosphorylierung in MDA-MB-231 Zellen den Gs-gekoppelten Rezeptoren zugeordnet werden. $A_{2B}AR$ stimuliert auch eine Ca^{2+}-Antwort, die mit der Angiogenese in Verbindung gebracht wird (Kohn et al., 1995). Interessanterweise ist das Wachstum von MDA-MB-231 Zellen mit nanomolare NECA Konzentration gehemmt, wobei diese in der Ca^{2+}-Mobilisierung inaktiv ist, so dass das Wachstum gehemmt werden kann, ohne dabei die Angiogenese zu fördern. LUF6210 ruft kein Ca^{2+} Signal hervor und ist daher von Bedeutung, wenn man bedenkt, dass es die ERK1/2 Phosphorylierung redurziert aber die Angiogenese nicht beeinflusst. LUF6210 ist deshalb therapeutisch ansprechend in der Behandlung von Krebs. Angesichts der Tatsache, dass die Stimulation der AC die Reduktion der ERK1/2-Phosphorylierung und der Proliferation in Krebszellen aktiviert, sind selective Gs-AC-PKA Agonisten erforderlich in der Therapie verschiedener maligner Erkrankungen.

8   References

Aguirre-Ghiso, J.A. (2007). Models, mechanisms and clinical evidence for cancer dormancy. Nat Rev Cancer 7, 834-846.

Albasanz, J.L., Rodriguez, A., Ferrer, I., and Martin, M. (2007). Up-regulation of adenosine A1 receptors in frontal cortex from Pick's disease cases. Eur J Neurosci 26, 3501-3508.

Antonioli, L., Blandizzi, C., Malavasi, F., Ferrari, D., and Hasko, G. (2016). Anti-CD73 immunotherapy: A viable way to reprogram the tumor microenvironment. Oncoimmunology 5, e1216292.

Antonioli, L., Blandizzi, C., Pacher, P., and Hasko, G. (2013a). Immunity, inflammation and cancer: a leading role for adenosine. Nat Rev Cancer 13, 842-857.

Antonioli, L., Pacher, P., Vizi, E.S., and Hasko, G. (2013b). CD39 and CD73 in immunity and inflammation. Trends Mol Med 19, 355-367.

Azzi, M., Charest, P.G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M., and Pineyro, G. (2003). Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. Proc Natl Acad Sci U S A 100, 11406-11411.

Baker, J.G., Hall, I.P., and Hill, S.J. (2003). Agonist and inverse agonist actions of beta-blockers at the human beta 2-adrenoceptor provide evidence for agonist-directed signaling. Mol Pharmacol 64, 1357-1369.

Ballarin, M., Fredholm, B.B., Ambrosio, S., and Mahy, N. (1991). Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. Acta Physiol Scand 142, 97-103.

Belardinelli, L., Shryock, J.C., Song, Y., Wang, D., and Srinivas, M. (1995). Ionic basis of the electrophysiological actions of adenosine on cardiomyocytes. FASEB J 9, 359-365.

Berne, R.M. (1963). Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. Am J Physiol 204, 317-322.

Bhalla, U.S., Ram, P.T., and Iyengar, R. (2002). MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. Science 297, 1018-1023.

Boeynaems, J.M., Communi, D., Gonzalez, N.S., and Robaye, B. (2005). Overview of the P2 receptors. Semin Thromb Hemost 31, 139-149.

Borea, P.A., Gessi, S., Merighi, S., and Varani, K. (2016). Adenosine as a Multi-Signalling Guardian Angel in Human Diseases: When, Where and How Does it Exert its Protective Effects? Trends Pharmacol Sci 37, 419-434.

Borea, P.A., Gessi, S., Merighi, S., Vincenzi, F., and Varani, K. (2017). Pathological overproduction: the bad side of adenosine. Br J Pharmacol 174, 1945-1960.

Brondello, J.M., Pouyssegur, J., and McKenzie, F.R. (1999). Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. Science 286, 2514-2517.

Burnstock, G. (1978). A basis for distinguishing two types of purinergic receptor Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approached. Straub, R.W. & Bolis, L. pp. 107–118.New York: Raven Press

Burnstock, G. (2007). Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 87, 659-797.

Caunt, C.J., and Keyse, S.M. (2013). Dual-specificity MAP kinase phosphatases (MKPs): shaping the outcome of MAP kinase signalling. FEBS J 280, 489-504.

Cekic, C., Sag, D., Li, Y., Theodorescu, D., Strieter, R.M., and Linden, J. (2012). Adenosine A2B receptor blockade slows growth of bladder and breast tumors. J Immunol *188*, 198-205.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37-40.

Cheng, Y., and Prusoff, W.H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol *22*, 3099-3108.

Chu, Y., Solski, P.A., Khosravi-Far, R., Der, C.J., and Kelly, K. (1996). The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. J Biol Chem *271*, 6497-6501.

Costa, T., and Herz, A. (1989). Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc Natl Acad Sci U S A *86*, 7321-7325.

Coulthard, L.R., White, D.E., Jones, D.L., McDermott, M.F., and Burchill, S.A. (2009). p38(MAPK): stress responses from molecular mechanisms to therapeutics. Trends Mol Med *15*, 369-379.

Crowell, S., Wancket, L.M., Shakibi, Y., Xu, P., Xue, J., Samavati, L., Nelin, L.D., and Liu, Y. (2014). Post-translational regulation of mitogen-activated protein kinase phosphatase (MKP)-1 and MKP-2 in macrophages following lipopolysaccharide stimulation: the role of the C termini of the phosphatases in determining their stability. J Biol Chem *289*, 28753-28764.

Cuadrado, A., and Nebreda, A.R. (2010). Mechanisms and functions of p38 MAPK signalling. Biochem J *429*, 403-417.

Dash, P.K., Karl, K.A., Colicos, M.A., Prywes, R., and Kandel, E.R. (1991). cAMP response element-binding protein is activated by Ca2+/calmodulin- as well as cAMP-dependent protein kinase. Proc Natl Acad Sci U S A *88*, 5061-5065.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J *351*, 95-105.

Davis, R.J. (1994). MAPKs: new JNK expands the group. Trends Biochem Sci *19*, 470-473.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell *103*, 239-252.

De Cesare, D., Fimia, G.M., and Sassone-Corsi, P. (1999). Signaling routes to CREM and CREB: plasticity in transcriptional activation. Trends Biochem Sci *24*, 281-285.

de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A., and Bos, J.L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature *396*, 474-477.

Decking, U.K., Schlieper, G., Kroll, K., and Schrader, J. (1997). Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. Circ Res *81*, 154-164.

Delghandi, M.P., Johannessen, M., and Moens, U. (2005). The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. Cell Signal *17*, 1343-1351.

DeNicola, G.F., Martin, E.D., Chaikuad, A., Bassi, R., Clark, J., Martino, L., Verma, S., Sicard, P., Tata, R., Atkinson, R.A., *et al.* (2013). Mechanism and consequence of the autoactivation of p38alpha mitogen-activated protein kinase promoted by TAB1. Nat Struct Mol Biol *20*, 1182-1190.

Dhillon, A.S., Pollock, C., Steen, H., Shaw, P.E., Mischak, H., and Kolch, W. (2002). Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. Mol Cell Biol *22*, 3237-3246.

Dickinson, R.J., and Keyse, S.M. (2006). Diverse physiological functions for dual-specificity MAP kinase phosphatases. J Cell Sci *119*, 4607-4615.

Drury, A.N., and Szent-Gyorgyi, A. (1929). The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. J Physiol *68*, 213-237.

Du, X., Ou, X., Song, T., Zhang, W., Cong, F., Zhang, S., and Xiong, Y. (2015). Adenosine A2B receptor stimulates angiogenesis by inducing VEGF and eNOS in human microvascular endothelial cells. Exp Biol Med (Maywood) *240*, 1472-1479.

Dubey, R.K., Gillespie, D.G., Mi, Z., and Jackson, E.K. (2005). Adenosine inhibits PDGF-induced growth of human glomerular mesangial cells via A(2B) receptors. Hypertension *46*, 628-634.

Dubey, R.K., Gillespie, D.G., Zacharia, L.C., Mi, Z., and Jackson, E.K. (2001). A(2b) receptors mediate the antimitogenic effects of adenosine in cardiac fibroblasts. Hypertension *37*, 716-721.

Dumaz, N., and Marais, R. (2003). Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. J Biol Chem *278*, 29819-29823.

Dumaz, N., and Marais, R. (2005a). Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. FEBS J *272*, 3491-3504.

Dumaz, N., and Marais, R. (2005b). Raf phosphorylation: one step forward and two steps back. Mol Cell *17*, 164-166.

Ebisuya, M., Kondoh, K., and Nishida, E. (2005). The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. J Cell Sci *118*, 2997-3002.

Eckle, T., Kewley, E.M., Brodsky, K.S., Tak, E., Bonney, S., Gobel, M., Anderson, D., Glover, L.E., Riegel, A.K., Colgan, S.P., *et al*. (2014). Identification of hypoxia-inducible factor HIF-1A as transcriptional regulator of the A2B adenosine receptor during acute lung injury. J Immunol *192*, 1249-1256.

Englert, M., Quitterer, U., and Klotz, K.N. (2002). Effector coupling of stably transfected human A3 adenosine receptors in CHO cells. Biochem Pharmacol *64*, 61-65.

Feoktistov, I., Goldstein, A.E., Ryzhov, S., Zeng, D., Belardinelli, L., Voyno-Yasenetskaya, T., and Biaggioni, I. (2002). Differential expression of adenosine receptors in human endothelial cells: role of A2B receptors in angiogenic factor regulation. Circ Res *90*, 531-538.

Feoktistov, I., Ryzhov, S., Zhong, H., Goldstein, A.E., Matafonov, A., Zeng, D., and Biaggioni, I. (2004). Hypoxia modulates adenosine receptors in human endothelial and smooth muscle cells toward an A2B angiogenic phenotype. Hypertension *44*, 649-654.

Fernandez-Gallardo, M., Gonzalez-Ramirez, R., Sandoval, A., Felix, R., and Monjaraz, E. (2016). Adenosine Stimulate Proliferation and Migration in Triple Negative Breast Cancer Cells. PLoS One *11*, e0167445.

Fimia, G.M., and Sassone-Corsi, P. (2001). Cyclic AMP signalling. J Cell Sci *114*, 1971-1972.

Fishman, P., Bar-Yehuda, S., Ardon, E., Rath-Wolfson, L., Barrer, F., Ochaion, A., and Madi, L. (2003). Targeting the A3 adenosine receptor for cancer therapy:

inhibition of prostate carcinoma cell growth by A3AR agonist. Anticancer Res *23*, 2077-2083.

Fishman, P., Bar-Yehuda, S., Madi, L., and Cohn, I. (2002). A3 adenosine receptor as a target for cancer therapy. Anticancer Drugs *13*, 437-443.

Fishman, P., Bar-Yehuda, S., Synowitz, M., Powell, J.D., Klotz, K.N., Gessi, S., and Borea, P.A. (2009). Adenosine receptors and cancer. Handb Exp Pharmacol, 399-441.

Fredholm, B.B., Irenius, E., Kull, B., and Schulte, G. (2001). Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. Biochem Pharmacol *61*, 443-448.

Garrington, T.P., and Johnson, G.L. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol *11*, 211-218.

Ge, B., Gram, H., Di Padova, F., Huang, B., New, L., Ulevitch, R.J., Luo, Y., and Han, J. (2002). MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science *295*, 1291-1294.

Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W.H., and Montminy, M.R. (1991). Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. Mol Cell Biol *11*, 1306-1312.

Gonzalez, G.A., and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell *59*, 675-680.

Gorzalka, S., Vittori, S., Volpini, R., Cristalli, G., von Kugelgen, I., and Muller, C.E. (2005). Evidence for the functional expression and pharmacological characterization of adenine receptors in native cells and tissues. Mol Pharmacol *67*, 955-964.

Grenz, A., Osswald, H., Eckle, T., Yang, D., Zhang, H., Tran, Z.V., Klingel, K., Ravid, K., and Eltzschig, H.K. (2008). The reno-vascular A2B adenosine receptor protects the kidney from ischemia. PLoS Med *5*, e137.

Hafner, S., Adler, H.S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994). Mechanism of inhibition of Raf-1 by protein kinase A. Mol Cell Biol *14*, 6696-6703.

Hajiahmadi, S., Panjehpour, M., Aghaei, M., and Shabani, M. (2015). Activation of A2b adenosine receptor regulates ovarian cancer cell growth: involvement of Bax/Bcl-2 and caspase-3. Biochem Cell Biol *93*, 321-329.

Hamilton, M., Liao, J., Cathcart, M.K., and Wolfman, A. (2001). Constitutive association of c-N-Ras with c-Raf-1 and protein kinase C epsilon in latent signaling modules. J Biol Chem *276*, 29079-29090.

Hansen, T.O., Rehfeld, J.F., and Nielsen, F.C. (2000). Cyclic AMP-induced neuronal differentiation via activation of p38 mitogen-activated protein kinase. J Neurochem *75*, 1870-1877.

Hinz, S., Lacher, S.K., Seibt, B.F., and Muller, C.E. (2014). BAY60-6583 acts as a partial agonist at adenosine A2B receptors. J Pharmacol Exp Ther *349*, 427-436.

Hoeren, M., Brawek, B., Mantovani, M., Loffler, M., Steffens, M., van Velthoven, V., and Feuerstein, T.J. (2008). Partial agonism at the human alpha(2A)-autoreceptor: role of binding duration. Naunyn Schmiedebergs Arch Pharmacol *378*, 17-26.

Hsueh, Y.P., and Lai, M.Z. (1995). c-Jun N-terminal kinase but not mitogen-activated protein kinase is sensitive to cAMP inhibition in T lymphocytes. J Biol Chem *270*, 18094-18098.

Hu, J.H., Chen, T., Zhuang, Z.H., Kong, L., Yu, M.C., Liu, Y., Zang, J.W., and Ge, B.X. (2007). Feedback control of MKP-1 expression by p38. Cell Signal *19*, 393-400.

Hutchinson, S.A., and Scammells, P.J. (2004). A(1) adenosine receptor agonists: medicinal chemistry and therapeutic potential. Curr Pharm Des *10*, 2021-2039.

Illiano, M., Sapio, L., Salzillo, A., Capasso, L., Caiafa, I., Chiosi, E., Spina, A., and Naviglio, S. (2018). Forskolin improves sensitivity to doxorubicin of triple negative breast cancer cells via Protein Kinase A-mediated ERK1/2 inhibition. Biochem Pharmacol *152*, 104-113.

Jacobson, K.A., Deflorian, F., Mishra, S., and Costanzi, S. (2011). Pharmacochemistry of the platelet purinergic receptors. Purinergic Signal *7*, 305-324.

Jacobson, K.A., and Gao, Z.G. (2006). Adenosine receptors as therapeutic targets. Nat Rev Drug Discov *5*, 247-264.

Jacobson, K.A., Merighi, S., Varani, K., Borea, P.A., Baraldi, S., Aghazadeh Tabrizi, M., Romagnoli, R., Baraldi, P.G., Ciancetta, A., Tosh, D.K.*, et al.* (2018). A3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy. Med Res Rev *38*, 1031-1072.

Johnson, G.L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science *298*, 1911-1912.

Johnson, G.L., and Nakamura, K. (2007). The c-jun kinase/stress-activated pathway: regulation, function and role in human disease. Biochim Biophys Acta *1773*, 1341-1348.

Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D.E., and Graybiel, A.M. (1998). A family of cAMP-binding proteins that directly activate Rap1. Science *282*, 2275-2279.

Kenakin, T. (2001). Inverse, protean, and ligand-selective agonism: matters of receptor conformation. FASEB J *15*, 598-611.

Kenakin, T. (2005). New concepts in drug discovery: collateral efficacy and permissive antagonism. Nat Rev Drug Discov *4*, 919-927.

Kenakin, T., and Miller, L.J. (2010). Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. Pharmacol Rev *62*, 265-304.

Keyse, S.M. (2000). Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. Curr Opin Cell Biol *12*, 186-192.

King, A.E., Ackley, M.A., Cass, C.E., Young, J.D., and Baldwin, S.A. (2006). Nucleoside transporters: from scavengers to novel therapeutic targets. Trends Pharmacol Sci *27*, 416-425.

King, A.J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S., and Marshall, M.S. (1998). The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature *396*, 180-183.

Klotz, K.N., Cristalli, G., Grifantini, M., Vittori, S., and Lohse, M.J. (1985). Photoaffinity labeling of A1-adenosine receptors. J Biol Chem *260*, 14659-14664.

Klotz, K.N., Hessling, J., Hegler, J., Owman, C., Kull, B., Fredholm, B.B., and Lohse, M.J. (1998). Comparative pharmacology of human adenosine receptor subtypes - characterization of stably transfected receptors in CHO cells. Naunyn Schmiedebergs Arch Pharmacol *357*, 1-9.

Knospe, M., Muller, C.E., Rosa, P., Abdelrahman, A., von Kugelgen, I., Thimm, D., and Schiedel, A.C. (2013). The rat adenine receptor: pharmacological characterization and mutagenesis studies to investigate its putative ligand binding site. Purinergic Signal *9*, 367-381.

Kochanek, P.M., Vagni, V.A., Janesko, K.L., Washington, C.B., Crumrine, P.K., Garman, R.H., Jenkins, L.W., Clark, R.S., Homanics, G.E., Dixon, C.E., *et al.* (2006). Adenosine A1 receptor knockout mice develop lethal status epilepticus after experimental traumatic brain injury. J Cereb Blood Flow Metab *26*, 565-575.

Koeppen, M., Eckle, T., and Eltzschig, H.K. (2011). The hypoxia-inflammation link and potential drug targets. Curr Opin Anaesthesiol *24*, 363-369.

Kohn, E.C., Alessandro, R., Spoonster, J., Wersto, R.P., and Liotta, L.A. (1995). Angiogenesis: role of calcium-mediated signal transduction. Proc Natl Acad Sci U S A *92*, 1307-1311.

Kull, B., Svenningsson, P., and Fredholm, B.B. (2000). Adenosine A(2A) receptors are colocalized with and activate g(olf) in rat striatum. Mol Pharmacol *58*, 771-777.

Kwak, S.P., Hakes, D.J., Martell, K.J., and Dixon, J.E. (1994). Isolation and characterization of a human dual specificity protein-tyrosine phosphatase gene. J Biol Chem *269*, 3596-3604.

Kyriakis, J.M., and Avruch, J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. J Biol Chem *271*, 24313-24316.

Kyriakis, J.M., and Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev *81*, 807-869.

Linden, J. (2005). Adenosine in tissue protection and tissue regeneration. Mol Pharmacol *67*, 1385-1387.

Linden, J., Thai, T., Figler, H., Jin, X., and Robeva, A.S. (1999). Characterization of human A(2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells. Mol Pharmacol *56*, 705-713.

Ma, D.F., Kondo, T., Nakazawa, T., Niu, D.F., Mochizuki, K., Kawasaki, T., Yamane, T., and Katoh, R. (2010). Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells. Hum Pathol *41*, 1550-1557.

Madi, L., Bar-Yehuda, S., Barer, F., Ardon, E., Ochaion, A., and Fishman, P. (2003). A3 adenosine receptor activation in melanoma cells: association between receptor fate and tumor growth inhibition. J Biol Chem *278*, 42121-42130.

Madi, L., Ochaion, A., Rath-Wolfson, L., Bar-Yehuda, S., Erlanger, A., Ohana, G., Harish, A., Merimski, O., Barer, F., and Fishman, P. (2004). The A3 adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. Clin Cancer Res *10*, 4472-4479.

Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. Science *298*, 1912-1934.

Mason, C.S., Springer, C.J., Cooper, R.G., Superti-Furga, G., Marshall, C.J., and Marais, R. (1999). Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J *18*, 2137-2148.

Matsuoka, M., and Igisu, H. (1998). Activation of c-Jun NH2-terminal kinase (JNK/SAPK) in LLC-PK1 cells by cadmium. Biochem Biophys Res Commun *251*, 527-532.

Merighi, S., Benini, A., Mirandola, P., Gessi, S., Varani, K., Leung, E., MacLennan, S., Baraldi, P.G., and Borea, P.A. (2005). A3 adenosine receptors modulate hypoxia-inducible factor-1alpha expression in human A375 melanoma cells. Neoplasia *7*, 894-903.

Merighi, S., Mirandola, P., Milani, D., Varani, K., Gessi, S., Klotz, K.N., Leung, E., Baraldi, P.G., and Borea, P.A. (2002). Adenosine receptors as mediators of both

cell proliferation and cell death of cultured human melanoma cells. J Invest Dermatol *119*, 923-933.

Merighi, S., Mirandola, P., Varani, K., Gessi, S., Leung, E., Baraldi, P.G., Tabrizi, M.A., and Borea, P.A. (2003). A glance at adenosine receptors: novel target for antitumor therapy. Pharmacol Ther *100*, 31-48.

Merighi, S., Varani, K., Gessi, S., Cattabriga, E., Iannotta, V., Ulouglu, C., Leung, E., and Borea, P.A. (2001). Pharmacological and biochemical characterization of adenosine receptors in the human malignant melanoma A375 cell line. Br J Pharmacol *134*, 1215-1226.

Mirza, A., Basso, A., Black, S., Malkowski, M., Kwee, L., Pachter, J.A., Lachowicz, J.E., Wang, Y., and Liu, S. (2005). RNA interference targeting of A1 receptor-overexpressing breast carcinoma cells leads to diminished rates of cell proliferation and induction of apoptosis. Cancer Biol Ther *4*, 1355-1360.

Moosavi, S.M., Prabhala, P., and Ammit, A.J. (2017). Role and regulation of MKP-1 in airway inflammation. Respir Res *18*, 154.

Mori Sequeiros Garcia, M., Gorostizaga, A., Brion, L., Gonzalez-Calvar, S.I., and Paz, C. (2015). cAMP-activated Nr4a1 expression requires ERK activity and is modulated by MAPK phosphatase-1 in MA-10 Leydig cells. Mol Cell Endocrinol *408*, 45-52.

Morrison, R.R., Teng, B., Oldenburg, P.J., Katwa, L.C., Schnermann, J.B., and Mustafa, S.J. (2006). Effects of targeted deletion of A1 adenosine receptors on postischemic cardiac function and expression of adenosine receptor subtypes. Am J Physiol Heart Circ Physiol *291*, H1875-1882.

Morton, S., Davis, R.J., McLaren, A., and Cohen, P. (2003). A reinvestigation of the multisite phosphorylation of the transcription factor c-Jun. EMBO J *22*, 3876-3886.

Nishihara, H., Hwang, M., Kizaka-Kondoh, S., Eckmann, L., and Insel, P.A. (2004). Cyclic AMP promotes cAMP-responsive element-binding protein-dependent induction of cellular inhibitor of apoptosis protein-2 and suppresses apoptosis of colon cancer cells through ERK1/2 and p38 MAPK. J Biol Chem *279*, 26176-26183.

Ohana, G., Bar-Yehuda, S., Arich, A., Madi, L., Dreznick, Z., Rath-Wolfson, L., Silberman, D., Slosman, G., and Fishman, P. (2003). Inhibition of primary colon carcinoma growth and liver metastasis by the A3 adenosine receptor agonist CF101. Br J Cancer *89*, 1552-1558.

Ohta, A., Gorelik, E., Prasad, S.J., Ronchese, F., Lukashev, D., Wong, M.K., Huang, X., Caldwell, S., Liu, K., Smith, P., *et al.* (2006). A2A adenosine receptor protects tumors from antitumor T cells. Proc Natl Acad Sci U S A *103*, 13132-13137.

Osinski, M.T., and Schror, K. (2000). Inhibition of platelet-derived growth factor-induced mitogenesis by phosphodiesterase 3 inhibitors: role of protein kinase A in vascular smooth muscle cell mitogenesis. Biochem Pharmacol *60*, 381-387.

Owens, D.M., and Keyse, S.M. (2007). Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. Oncogene *26*, 3203-3213.

Panjehpour, M., Castro, M., and Klotz, K.N. (2005). Human breast cancer cell line MDA-MB-231 expresses endogenous A(2B) adenosine receptors mediating a Ca2+ signal. Brit J Pharmacol *145*, 211-218.

Panjehpour, M., and Karami-Tehrani, F. (2007). Adenosine modulates cell growth in the human breast cancer cells via adenosine receptors. Oncol Res *16*, 575-585.

Pastor-Anglada, M., Urtasun, N., and Perez-Torras, S. (2018). Intestinal Nucleoside Transporters: Function, Expression, and Regulation. Compr Physiol *8*, 1003-1017.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev *22*, 153-183.

Pedata, F., Corsi, C., Melani, A., Bordoni, F., and Latini, S. (2001). Adenosine extracellular brain concentrations and role of A2A receptors in ischemia. Ann N Y Acad Sci *939*, 74-84.

Pedram, A., Razandi, M., and Levin, E.R. (1998). Extracellular signal-regulated protein kinase/Jun kinase cross-talk underlies vascular endothelial cell growth factor-induced endothelial cell proliferation. J Biol Chem *273*, 26722-26728.

Penn, R.B., Parent, J.L., Pronin, A.N., Panettieri, R.A., Jr., and Benovic, J.L. (1999). Pharmacological inhibition of protein kinases in intact cells: antagonism of beta adrenergic receptor ligand binding by H-89 reveals limitations of usefulness. J Pharmacol Exp Ther *288*, 428-437.

Plotnikov, A., Zehorai, E., Procaccia, S., and Seger, R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim Biophys Acta *1813*, 1619-1633.

Rahman, A., Anwar, K.N., Minhajuddin, M., Bijli, K.M., Javaid, K., True, A.L., and Malik, A.B. (2004). cAMP targeting of p38 MAP kinase inhibits thrombin-induced NF-kappaB activation and ICAM-1 expression in endothelial cells. Am J Physiol Lung Cell Mol Physiol *287*, L1017-1024.

Rajagopal, S., Rajagopal, K., and Lefkowitz, R.J. (2010). Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov *9*, 373-386.

Ralevic, V., and Burnstock, G. (1991). Effects of purines and pyrimidines on the rat mesenteric arterial bed. Circ Res *69*, 1583-1590.

Raman, M., Chen, W., and Cobb, M.H. (2007). Differential regulation and properties of MAPKs. Oncogene *26*, 3100-3112.

Rao, G.N., and Runge, M.S. (1996). Cyclic AMP inhibition of thrombin-induced growth in vascular smooth muscle cells correlates with decreased JNK1 activity and c-Jun expression. J Biol Chem *271*, 20805-20810.

Reid, E.A., Kristo, G., Yoshimura, Y., Ballard-Croft, C., Keith, B.J., Mentzer, R.M., Jr., and Lasley, R.D. (2005). In vivo adenosine receptor preconditioning reduces myocardial infarct size via subcellular ERK signaling. Am J Physiol Heart Circ Physiol *288*, H2253-2259.

Rizvi, Y.Q., Mehta, C.S., and Oyekan, A. (2013). Interactions of PPAR-alpha and adenosine receptors in hypoxia-induced angiogenesis. Vascul Pharmacol *59*, 144-151.

Rogel, A., Bromberg, Y., Sperling, O., and Zoref-Shani, E. (2005). Phospholipase C is involved in the adenosine-activated signal transduction pathway conferring protection against iodoacetic acid-induced injury in primary rat neuronal cultures. Neurosci Lett *373*, 218-221.

Rosentreter U, Henning R, Bauser M, Krämer T, Vaupel A, Hübsch W, Dembowsky K, Salcher-Schrauf-Stätter O, Stasch JP, Krahn T and Perzborn E. Inventors, Bayer, assignee. Substituted 2-thio-3,5-dicyano-4-aryl-6-aminopyridines and the use thereof as adenosine receptor ligands. WO Patent 2001/025210.

Roskoski, R., Jr. (2012a). ERK1/2 MAP kinases: structure, function, and regulation. Pharmacol Res *66*, 105-143.

Roskoski, R., Jr. (2012b). MEK1/2 dual-specificity protein kinases: structure and regulation. Biochem Biophys Res Commun *417*, 5-10.

Ruppert, C., Deiss, K., Herrmann, S., Vidal, M., Oezkur, M., Gorski, A., Weidemann, F., Lohse, M.J., and Lorenz, K. (2013). Interference with ERK(Thr188) phosphorylation impairs pathological but not physiological cardiac hypertrophy. Proc Natl Acad Sci U S A *110*, 7440-7445.

Ryser, S., Tortola, S., van Haasteren, G., Muda, M., Li, S., and Schlegel, W. (2001). MAP kinase phosphatase-1 gene transcription in rat neuroendocrine cells is modulated by a calcium-sensitive block to elongation in the first exon. J Biol Chem *276*, 33319-33327.

Sands, W.A., and Palmer, T.M. (2008). Regulating gene transcription in response to cyclic AMP elevation. Cell Signal *20*, 460-466.

Saxena, M., and Mustelin, T. (2000). Extracellular signals and scores of phosphatases: all roads lead to MAP kinase. Semin Immunol *12*, 387-396.

Schmid, E., Neef, S., Berlin, C., Tomasovic, A., Kahlert, K., Nordbeck, P., Deiss, K., Denzinger, S., Herrmann, S., Wettwer, E., *et al.* (2015). Cardiac RKIP induces a beneficial beta-adrenoceptor-dependent positive inotropy. Nat Med *21*, 1298-1306.

Schulte, G., and Fredholm, B.B. (2000). Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. Mol Pharmacol *58*, 477-482.

Schulte, G., and Fredholm, B.B. (2002). Signaling pathway from the human adenosine A(3) receptor expressed in Chinese hamster ovary cells to the extracellular signal-regulated kinase 1/2. Mol Pharmacol *62*, 1137-1146.

Schulte, G., and Fredholm, B.B. (2003). Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal *15*, 813-827.

Sevetson, B.R., Kong, X., and Lawrence, J.C., Jr. (1993). Increasing cAMP attenuates activation of mitogen-activated protein kinase. Proc Natl Acad Sci U S A *90*, 10305-10309.

Sheng, M., McFadden, G., and Greenberg, M.E. (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. Neuron *4*, 571-582.

Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252*, 1427-1430.

Sheth, S., Brito, R., Mukherjea, D., Rybak, L.P., and Ramkumar, V. (2014). Adenosine receptors: expression, function and regulation. Int J Mol Sci *15*, 2024-2052.

Slotkin, T.A., Zhang, J., Dancel, R., Garcia, S.J., Willis, C., and Seidler, F.J. (2000). Beta-adrenoceptor signaling and its control of cell replication in MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat *60*, 153-166.

Spychala, J. (2000). Tumor-promoting functions of adenosine. Pharmacol Ther *87*, 161-173.

Stemmer, S.M., Benjaminov, O., Medalia, G., Ciuraru, N.B., Silverman, M.H., Bar-Yehuda, S., Fishman, S., Harpaz, Z., Farbstein, M., Cohen, S., *et al.* (2013). CF102 for the treatment of hepatocellular carcinoma: a phase I/II, open-label, dose-escalation study. Oncologist *18*, 25-26.

Stringaris, A.K., Geisenhainer, J., Bergmann, F., Balshusemann, C., Lee, U., Zysk, G., Mitchell, T.J., Keller, B.U., Kuhnt, U., Gerber, J., *et al.* (2002). Neurotoxicity of pneumolysin, a major pneumococcal virulence factor, involves calcium influx and depends on activation of p38 mitogen-activated protein kinase. Neurobiol Dis *11*, 355-368.

Sun, C.X., Zhong, H., Mohsenin, A., Morschl, E., Chunn, J.L., Molina, J.G., Belardinelli, L., Zeng, D., and Blackburn, M.R. (2006). Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. J Clin Invest *116*, 2173-2182.

Sunahara, R.K., Dessauer, C.W., and Gilman, A.G. (1996). Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol *36*, 461-480.

Synnestvedt, K., Furuta, G.T., Comerford, K.M., Louis, N., Karhausen, J., Eltzschig, H.K., Hansen, K.R., Thompson, L.F., and Colgan, S.P. (2002). Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. J Clin Invest *110*, 993-1002.

Synowitz, M., Glass, R., Farber, K., Markovic, D., Kronenberg, G., Herrmann, K., Schnermann, J., Nolte, C., van Rooijen, N., Kiwit, J., *et al.* (2006). A1 adenosine receptors in microglia control glioblastoma-host interaction. Cancer Res *66*, 8550-8557.

Tasken, K., and Aandahl, E.M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev *84*, 137-167.

Tawfik, H.E., Schnermann, J., Oldenburg, P.J., and Mustafa, S.J. (2005). Role of A1 adenosine receptors in regulation of vascular tone. Am J Physiol Heart Circ Physiol *288*, H1411-1416.

Urban, J.D., Clarke, W.P., von Zastrow, M., Nichols, D.E., Kobilka, B., Weinstein, H., Javitch, J.A., Roth, B.L., Christopoulos, A., Sexton, P.M., *et al.* (2007). Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther *320*, 1-13.

Valls, M.D., Cronstein, B.N., and Montesinos, M.C. (2009). Adenosine receptor agonists for promotion of dermal wound healing. Biochem Pharmacol *77*, 1117-1124.

van der Hoeven, D., Wan, T.C., Gizewski, E.T., Kreckler, L.M., Maas, J.E., Van Orman, J., Ravid, K., and Auchampach, J.A. (2011). A role for the low-affinity A2B adenosine receptor in regulating superoxide generation by murine neutrophils. J Pharmacol Exp Ther *338*, 1004-1012.

Violin, J.D., and Lefkowitz, R.J. (2007). Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci *28*, 416-422.

Wei, Q., Costanzi, S., Balasubramanian, R., Gao, Z.G., and Jacobson, K.A. (2013). A2B adenosine receptor blockade inhibits growth of prostate cancer cells. Purinergic Signal *9*, 271-280.

Weisman, G.A., Garrad, R.C., Erb, L.J., Otero, M., Gonzalez, F.A., and Clarke, L.L. (1998). Structure and function of P2Y2 nucleotide receptors in cystic fibrosis (CF) epithelium. Adv Exp Med Biol *431*, 417-424.

Wendler, C.C., Amatya, S., McClaskey, C., Ghatpande, S., Fredholm, B.B., and Rivkees, S.A. (2007). A1 adenosine receptors play an essential role in protecting the embryo against hypoxia. Proc Natl Acad Sci U S A *104*, 9697-9702.

Weston, C.R., and Davis, R.J. (2002). The JNK signal transduction pathway. Curr Opin Genet Dev *12*, 14-21.

Whitmarsh, A.J. (2007). Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. Biochim Biophys Acta *1773*, 1285-1298.

Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M.J., and Sturgill, T.W. (1993). Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. Science *262*, 1065-1069.

Yang, D., Koupenova, M., McCrann, D.J., Kopeikina, K.J., Kagan, H.M., Schreiber, B.M., and Ravid, K. (2008). The A2b adenosine receptor protects against vascular injury. Proc Natl Acad Sci U S A *105*, 792-796.

Yang, S.H., Whitmarsh, A.J., Davis, R.J., and Sharrocks, A.D. (1998). Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. EMBO J *17*, 1740-1749.

Young, J.D. (2016). The SLC28 (CNT) and SLC29 (ENT) nucleoside transporter families: a 30-year collaborative odyssey. Biochem Soc Trans *44*, 869-876.

Yu, Z.P., Matsuoka, M., Wispriyono, B., Iryo, Y., and Igisu, H. (2000). Activation of mitogen-activated protein kinases by tributyltin in CCRF-CEM cells: role of intracellular Ca(2+). Toxicol Appl Pharmacol *168*, 200-207.

Zetterstrom, T., Vernet, L., Ungerstedt, U., Tossman, U., Jonzon, B., and Fredholm, B.B. (1982). Purine levels in the intact rat brain. Studies with an implanted perfused hollow fibre. Neurosci Lett *29*, 111-115.

Zheng, C.F., and Guan, K.L. (1993). Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. J Biol Chem *268*, 11435-11439.

Zhou, H., Zheng, M., Chen, J., Xie, C., Kolatkar, A.R., Zarubin, T., Ye, Z., Akella, R., Lin, S., Goldsmith, E.J., *et al*. (2006). Determinants that control the specific interactions between TAB1 and p38alpha. Mol Cell Biol *26*, 3824-3834.

Zimmermann, S., and Moelling, K. (1999). Phosphorylation and regulation of Raf by Akt (protein kinase B). Science *286*, 1741-1744.

9 Abbreviations

AC	Adenylyl cyclase
ADPase	Adenosindiphosphatasen
AR	Adenosine receptor
ATF-1	Activating transcription factor 1
ATPase	Adenosintriphosphatasen
CF	Cystic fibrosis
CHO	Chinese hamster ovary
CHX	Cycloheximide
CRE	cAMP response element
CREB	cAMP responsive element-binding protein
CREM	cAMP response element modulator
DAG	Diacylglycerol
DUSP	Dual specificity protein phosphatase
EC_{50}	Half maximal effective concentration
E_{max}	Percent of the full response
Epac	Exchange factor protein activated by cAMP
ER	Estrogen receptor
ERK1/2	Extracellular signal regulated kinase1/2
FSK	Forskolin
GAPDH	Glyceraldehyde 3-phosphate-dehydrogenase
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor

HEK	Human embryonic kidney
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
IC_{50}	Half maximal inhibitory concentration
IP_3	Inositol 1,4,5-triphosphate
ISO	Isoproterenol
JNK	c-Jun-N-terminal kinase
K_i	Dissociation constant
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MEK	MAPK/ERK kinase
MEK1/2	Mitogen-activated protein kinase kinase 1/2
MKK	MAPK kinase
MKP-1	Mitogen-activated protein kinase phosphatase 1
MKP-2	Mitogen-activated protein kinase phosphatase 2
MKPs	Mitogen-activated protein kinase phosphatases
P38	Stress-activated protein kinase, 38 kDa
PAGE	Polyacrylamide gelelectrophoresis
pERK1/2	Phospho-extracellular signal regulated kinase1/2
PI3-Kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-diphosphate
PKA	cAMP-dependent protein kinase

PKC	Ca^{2+}-dependent protein kinase
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PR	Progesterone receptor
PTP	Protein thyrosine phosphatase
Rmax	Maximal fluorescence ratio
S.D	Standard deviation
SAH	S-adenosylhomocysteine
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
siRNA	Short interfering RNA
TAB-1	TAK-1-binding protein
TFs	Transcription factors
TNBC	Triple negative breast cancer
β-AR	β-adrenergic receptor

10 Curriculum Vitae

Language skills

French, German, English

Conference contributions

Oral Presentations

Koussémou M., Lorenz K., Kachler S., Klotz K.-N. (2013) Activation of multiple signaling pathways of the human A_{2B} adenosine receptor by structurally diverse agonists. Oral presentation at the 79th Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT-2013). 5-7 March 2013, Martin-Luther-University Halle-Wittenberg.

Koussémou M., Kachler S, Klotz K.-N. 2013. Multiple signaling pathways of the human A_{2B} adenosine receptor: Activation by structurally diverse agonists. Oral presentation at the 5th Joint Italian – German Purine Club Meeting "Fostering translational research on Purines by Italian-German joint efforts". 18-21 September 2013, Palazzo Ruffi-Briolini, Rimini, Italy. Purinergic Signal. 2014 Jun; 10(2): 369-417. doi: 10.1007/s11302-013-9389-9

Posters

Koussémou M., Lorenz K., Kachler S., Klotz K.-N. 2014. The A_{2B} adenosine receptor in MDA-MB-231 breast cancer cells mediates inhibition of ERK1/2 phosphorylation by activation of MAPK-phosphatase-1. Poster presented at the PURINES 2014: Nucleotides, Nucleosides and Nucleobases - International Conference on Signaling, Drugs and Targets. 23-27 July 2014, Rheinische Friedrich-Wilhelms-Universität Bonn, Germany. Purinergic Signal., 2014 Dec; 10(4): 657-854. Poster K 170 doi: 10.1007/s11302-014-9433-4

Koussémou M., Lorenz K., Kachler S., Klotz K.-N. 2014. The A_{2B} adenosine receptor in MDA-MB-231 breast cancer cells mediates inhibition of ERK1/2 phosphorylation by activation of MKP-1. Poster presented at **EUREKA!** 9th International Symposium organized by the Students of the GSLS, University of Würzburg

Koussémou M., Lorenz K., Kachler S., Klotz K.-N. 2015. Regulation of MAPK-signaling by A_{2B} adenosine receptors in MDA-MB-231 breast cancer cells. Poster presented at **6**[th] Joint German-Italian Purine Club Meeting. International Conference on Purinergic Signaling. 23-25 July, Hamburg

Publications

Koussémou M., Lorenz K., Klotz K.-N. (2018) The A_{2B} adenosine receptor in MDA-MB-231 breast cancer cells diminishes ERK1/2 phosphorylation by activation of MAPK-phosphatase-1. PlosOne *13*(8), e0202914

Koussémou M. & Klotz, KN. Agonists activate different A2B adenosine receptor signaling pathways in MBA-MD-231 breast cancer cells with distinct potencies. Naunyn-Schmiedeberg's Arch Pharmacol (2019). https://doi.org/10.1007/s00210-019-01695-2

Place, Date   Signature

Würzburg, 11.2.2019

11 Acknowledgements