# Rapid action of glucocorticoids in mast cells

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von

**Alice Bonicelli, Dipl.Vet.Med.**

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| | |
|---|---|
| KIT-Dekan: | Prof. Dr. Willem Klopper |
| Referent: | Prof. Dr. Andrew C.B. Cato |
| Korreferent: | Prof. Dr. Martin Bastmeyer |
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Alice Bonicelli, Dipl.Vet.Med.

Karlsruhe, 26th October 2016

# Zusammenfassung

Mastzellen spielen eine wichtige Rolle bei Allergien. Sie werden über hochaffine membranständige IgE Rezeptoren aktiviert und ihre Wirkung ist schnell durch Glucocorticoide (GC) gehemmt. Der genau Wirkmechanismus ist dabei noch nicht bekannt. In der hier vorgestellten Arbeit wurde der Effekt von GC auf IgE-aktivierte Mastzellen aus dem Knochenmark (bone marrow derived mast cells - BMMCs) und aus der Bauchhöhle (peritoneal cell-derived mast cells - PCMCs) untersucht. Es konnten zwei verschiedene inhibierende Effekte der GC auf die Mastzellen gezeigt werden. Zum Einen, eine 10 - 15 minütige Inhibition der Mastzell-Degranulation und zum Anderen, eine 30 - 60 minütige Inhibition der Phosphorylierung der Mitogen aktivierte Proteinkinases (MAPKs). Um dabei die Rolle des Glucocorticoidrezeptor (GR) zu untersuchen, wurde durch ein Cre/loxP Rekombinationssystem eine GR "knock-out" Mutante in PCMCs generiert. Der inhibierende Effekt der GC auf die Mastzell-Degranulation und die MAPKs Phosphorylierung konnte in der GR "Knock-out" Mutante aufgehoben werden. Dies weist darauf hin, dass die inhibierende Funktion des GC über den GR vermittelt wird. Andere Studien haben postuliert, dass die Lokalisation des GR in der Membran der Zielzelle für den Wirkmechanismus der GC von Bedeutung ist. In der hier vorgestellten Arbeit, die Membransrekrutierung des GR ist durch das Palmitoylierungs-Motiv (yl**c**mktllls) des Rezeptors zu auftreten vermutet. Um die Rolle des Motivs in der Lokalisation des GR zu untersuchen, wurde ein Cystein an Position 665 des Motivs gegen ein Alanin (C665A) ausgetauscht und so eine Mutante hergestellt. Die Mutante "C665A" wurde zusätlich mit einem grün floureszierenden Protein (GFP) fusioniert (GR-GFP MutC665A). Sowohl die grün-floureszierende GR-GFP MutC665A-Mutante als auch ein grün-floureszierender Wildtyp-GR (GR-GFP) wurden stabil in RBL-2H3 Mastzellen transfiziert und die Lokalisation in der Plasmamembran bestimmt. Dabei werden Allergene durch die "Polymer pen lithography" auf eine Glasfläche aufgebracht und die transfizierten Zellen durch Bindung an die Allergene immobilisiert. Die Rezeptor-Mutante zeigte im Vergleich zum Wildtyp-GR eine verminderte Rekrutierung des Rezeptors auf der Mastzell-Membran. Um den Mechanismus der Rezeptorrekrutierung noch genauer zu untersuchen, wurden Einzelmolekül-Tracking-Experimente durchgeführt. Dabei wurde ein GR Konstrukt benutzt, das an ein photoaktivierbares floureszentes Protein (mEos2-GR) gebunden ist. Dieses Konstrukt wurde transient in RBL-2H3 Zellen transfiziert und das interne Floureszenssignal mittels Interner Totalreflexionsfluoreszenzmikroskopie (TIRF) detektiert. Es konnte gezeigt werden, dass sich die GR proximal der Membran frei bewegen können. Diese Bewegung konnte durch Behandlung der Zellen mit GC verlangsamt werden. Die Aktivierung der Zellen allein oder in Kombination mit ihrer Behandlung mit GC konnte die Bewegung des Rezeptors auch verlangsamen. Diese dynamischen Veränderungen der GR könnten darauf hinweisen, dass der Rezeptor direkt mit Zellmembranproteinen oder mit membrannahen zytoplasmatischen Proteinen interagiert, und das diese Interaktion für den Wirkmechanismus von GC auf Mastzellen von Bedeutung ist.

# Abstract

Mast cells are important immune cells in allergy. They function through activation of membrane high-affinity immunoglobulin E (IgE) receptors and their action is rapidly suppressed by glucocorticoids (GCs) via an unknown mechanism. In the work presented here, rapid effect of GCs was investigated in IgE-activated mouse bone marrow derived mast cells (BMMCs) and mouse peritoneal cell-derived mast cells (PCMCs). Two rapid inhibitory effects of GCs were observed in these cells on degranulation and on mitogen-activated protein kinase (MAPKs) activity. First, a 10 - 15 min GC-mediated inhibitory effect on degranulation and second a 30 - 60 min effect on MAPKs phosphorylation. To investigate the contribution of the glucocorticoid receptor (GR) to these rapid effects of GCs, a knock-out of the GR was specifically generated in the PCMCs through Cre/loxP recombination system. The rapid effects of GCs on degranulation and MAPKs phosphorylation were abolished in the GR knock-out PCMCs, indicating that the receptor mediates these functions of the hormone. Rapid mechanism of action of GCs has been postulated to occur through membrane localization of GR via a putative palmitoylation motif (yl**c**mktllls). To determine the involvement of this motif in the membrane recruitment of the GR, the cysteine residue at amino acid 665 in the motif was mutated to an alanine (C665A). The mutant “C665A” GR fused to a green fluorescent protein (GR-GFP MutC665A) and the wild-type GR-GFP were stably transfected into RBL-2H3 mast cells and their localization to the plasma membrane was investigated. This study was made feasible by the immobilization of the transfected cells on allergens printed on a glass surface by the technique of polymer pen lithography. The mutant receptor showed an impaired recruitment to the plasma membrane compared to the wild-type receptor. To generate more knowledge on the recruitment process, single molecule tracking experiments were carried out using a GR construct fused to a photo-convertible fluorescent protein (mEos2-GR). This construct was transiently transfected into RBL-2H3 mast cells and total internal reflection microscopy (TIRF) was carried out. The GR was found to be motile in the proximity of the plasma membrane but its movement was slowed down upon treatment of the cells with GC. A reduced motion of the GR was also observed upon activation of the cells by IgE crosslinking alone or in combination with GC. These dynamic changes in motility of the GR near the plasma membrane suggest a possible interaction of the receptor with plasma membrane components or cytoplasmic proteins in proximity of the plasma membrane that may be of relevance to the rapid action of GCs in mast cells.

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# Abbreviations

**Table 0.1**: Abbreviations and their meaning

| Abbreviation | Meaning |
|---|---|
| µF | Microfarad |
| µW | Microwatt |
| A | Adenine |
| ACK | Ammonium-Chloride-Potassium |
| APS | Ammonium persulfate |
| ATP | Adenosintriphosphat |
| BMMCs | Bone marrow derived mast cells |
| bp | Basepare |
| BSA | Bovine serum albumin |
| C | Cytosine |
| $CaCl_2$ | Calcium chloride |
| CreTG | Cre transgenic mice |
| $CuSO_4$ | Copper (II) sulfate |
| Dex | Dexamethasone ($10^{-7}$ M) |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNP | 2,4-Dinitrophenol |
| DNP-BSA | Albumin from Bovine Serum, 2,4-Dinitrophenylated |
| dNTP | Desoxyribonucleoside triphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetra acetate |
| EGTA | Ethylene glycol tetraacetic acid |
| Erk | Extracellular regulated kinase |
| EtOH | Ethanol |

| Abbreviation | Meaning |
|---|---|
| FBS | Fetal bovine serum |
| FcεRI | High affinity IgE receptor |
| G | Guanine |
| GC | Glucocorticoid |
| GFP | Green fluorescent protein |
| GR | Glucocorticoid receptor |
| $GR^{flox/flox}$ | GR gene flanked by loxP sites (homozygous) |
| $GR^{flox/wt}$ | GR gene loxP-flanked and wild-type (heterozygous) |
| $GR^{wt/wt}$ | GR gene wild-type (homozygous) |
| h | Hour |
| HCl | Hydrogen chloride |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HRP | Horse radish peroxidase |
| HSP | Heat shock protein |
| IgE | Immunoglobulin E |
| IgG | Immunoglobulin G |
| IL-3 | Interleuchin-3 |
| IMDM | Iscove's Modified Dulbecco's Medium |
| JNK | Jun-amino-terminal kinase |
| $K_2HPO_4$ | Potassium phosphate dibasic |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Potassium phosphate monobasic |
| $KHCO_3$ | Potassium hydrogen carbonate |
| KO | Knock-out |
| L-glu | L-glutamine |
| Luc | Luciferase |
| M | Molar |
| MAPK | Mitogen-activated protein kinase |
| MetOH | Methanol |
| $MgCl_2$ | Magnesium chloride |

| Abbreviation | Meaning |
|---|---|
| $MgSO_4$ | Magnesium sulfate |
| mMCP | Mouse mast cell protease |
| MMTV-Luc | Mouse mammary tumor virus-Luciferase |
| ms | millisecond |
| $Na_2HPO_4$ | Disodium hydrogen phosphate |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| $NH_4Cl$ | Ammonium chloride |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCMCs | Peritoneal cell-derived mast cells |
| Pen/Strep | Penicillin/Streptomycin |
| pNAG | 4-Nitrophenyl N-acetyl-β-D-glucosaminide |
| PVDF | Polyvinylidene fluoride |
| pyr | Pyruvate |
| rcf | Relative centrifugal force |
| rpm | Revolutions per minute |
| RT | Room temperature |
| SAP | Stress-activated protein kinase |
| SCF | Stem cell factor |
| SDS | Sodium dodecyl sulfate |
| T | Thymine |
| TAE | Tris-acetate-EDTA |
| TBST | Tris-buffered saline with Tween-20 |
| TE | Tris-EDTA |
| TEMED | Tetramethylethylenediamine |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| U | Unit |
| V | Volt |
| WT | Wild-type |

# 1 INTRODUCTION

## 1.1 Glucocorticoids

### 1.1.1 Physiology

Glucocorticoids (GCs) such as cortisol are endogenous steroid hormones, originated from cholesterol, that regulate several physiological functions like glucose homeostasis, hydro-mineral balance, protein and lipid metabolism, skeletal growth, development, behavior and apoptosis [1]. In response to stress or osmotic perturbation [2], cortisol is secreted by the adrenal cortex through the hypothalamus-pituitary-adrenal axis [3] and released into the bloodstream. Within the blood, GCs can reach different organs and tissues like central nervous system, lungs, skin, intestines, muscles and kidneys. In the liver, for example, they mediate gluconeogenesis, with consequent hyper-glycemic effect associated with cellular reduced glucose uptake and mobilization of amino acids from extra-hepatic tissues [4]. In adipose tissue they exert lipolytic effect [5]. Moreover, GCs have been reported to exert immunosuppressive and strong anti-inflammatory effects [2, 6–10]. Due to these properties, synthetic GCs such as prednisolone or dexamethasone (Dex), are widely used as drugs in the therapy of inflammations, allergic reactions or autoimmune diseases [11]. Although their efficacy is high, their prolonged use in chronic diseases leads to several detrimental effects, such as osteoporosis, obesity, increased breakdown of skeletal muscle mass, thinning of the skin, delay of wound healing process, alopecia, immunodeficiency, fatty liver development and insulin resistant diabetes [12–14].
In order to overcome these side effects, the mechanism of action of GCs has to be studied in detail.

## 1.2 Mechanisms of action of glucocorticoids

GCs are lipophilic molecules that can easily diffuse from the blood through the cell membrane. Once inside cells, their action is classically mediated by their binding to the glucocorticoid receptor (GR).

### 1.2.1 The nuclear receptor superfamily and glucocorticoid receptor

The human GR (hGR) was cloned in 1985 and was then found to be part of a superfamily of nuclear receptors [15]. Different classifications have been proposed to describe this family of proteins [16, 17]. Mangelsdorf *et al.* (1995), for example, divided the receptors into four groups depending on the type of ligand they bind, the way they interact with DNA and their dimerization properties [16]. GR belongs to the Class I group of receptors called “steroid receptors” because they bind to steroid hormones. In addition to GR, this group also includes receptors for mineralocorticoid (MR), progesterone (PR), estrogen (ER) and androgen (AR). The Class II group comprises retinoid X receptor (RXR) heterodimers, to which the thyroid hormone receptor (TR), the retinoic acid receptor (RAR), vitamin D receptor (VDR), eicosanoids receptor (peroxisome proliferator-activated receptor, PPAR) and ecdysone receptor (EcR) belong. The Classes III and IV include homodimeric and monomeric receptors called “orphan receptors” because their ligand is still unknown [16, 18].

All these receptors, and in particular the steroid receptors, share a common structure that consists of three main functional domains: a variable N-terminal transactivation domain, a highly conserved DNA-binding domain (DBD) responsible to target the receptor to specific regions of the DNA called “hormone response elements” and a variable C-terminal domain where a well conserved ligand-binding domain (LBD) resides. A flexible hinge region typically containing the nuclear localization signal (NLS) connects the last two domains [15, 18, 19]. Two NLSs are present in the hGR: the classical one (NLS1) that resides in the area between the DBD and the hinge region and a second one (NLS2) located in the C-terminal domain of the receptor [4, 20]. The nuclear translocation of the GR requires the interaction between the classical NLS1 and cytoplasmic transport proteins called importins [21]. This interaction can occur both in a ligand-dependent and -independent manner [20, 22]. The NLS2 seems to have a weaker role in mediating the nuclear import of the receptor. Its activity is strictly dependent on the functionality of the NLS1 and on the binding of the hormone to the ligand binding domain where the NLS2 resides [23, 24]. Moreover, it has been discovered that the so called “Hsp90-based chaperone machinery” is also involved in nuclear import of the receptor [25]. This machinery consists of the interaction of Hsp90-recruited co-chaperones with microtubules associated molecular motors such as dynein [26, 27]. The GR is encoded by a gene named “NR3C1”, which is situated on chromosome 5 and is composed of 9 exons. Alternative splicing of exon 9 generates two isoforms of the receptor: the predominant GRα which binds endogenous GCs and the less expressed GRβ, which does not bind any GCs. Moreover, this latter receptor is transcriptionally inactive and is thought to behave as a dominant negative regulator of GRα [7, 28, 29]. At the mature messenger RNA (mRNA) level, exon 1 represents the 5’-untranslated region (UTR), exons 2 encodes the N-terminal domain and its activation function domain 1 (AF1) involved in the regulation of gene transcription in a ligand-independent manner [30]. Exons 3 and 4 encode the zinc fingers of the DNA-binding domain and finally exons 5 to 9 are code for the large ligand-binding domain, the activation function domain 2 (AF2) and the 3’-UTR of the protein [29, 31]. Figure 1.1 shows a schematic view of the described structure of the hGRα.

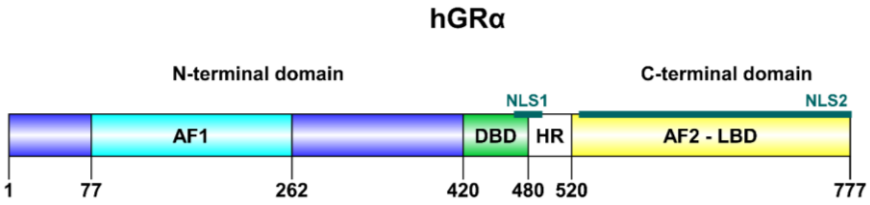


**Figure 1.1:** Schematic view of the structure of human GRα

In blue the N-terminal domain with its activation function 1 (AF1, light blue), in green the DNA-binding domain, in white the hinge region (HR) that harbors the nuclear localization signal (NLS1) and in yellow the C-terminal domain with the ligand-binding domain, the activation function 2 and another NLS (NLS2) activity. The numbers refer to the position of the amino acids. [*DOG 1.0: Illustrator of Protein Domain Structures (32)]*

Nearly all mammalian cells express the GR protein, but the GR-mediated expression of specific sets of GCs responsive genes depends on the tissue and the cell type [33].
In the inactive state, the GR is located in the cytoplasm, in a multi-protein complex that includes heat shock proteins (Hsp90, Hsp70, Hsp40 and others), p23 and co-chaperones such as FKBP52, FKBP51, Cyp40 [18, 34–39]. This complex, and in particular the chaperone Hsp90, helps to maintain the GR in a folded and hormone-responsive state [39]. Upon binding of the hormone, the receptor undergoes a conformational change that brings to its dissociation from most of the proteins of the complex and exposure of the NLSs. As a consequence, the GR translocates into the nucleus. As soon as the GR-GC complex reaches the nucleus, it gives rise to positive or negative regulation of target genes expression (“genomic mechanism of action of GCs”) [40].

### 1.2.2 Glucocorticoid-mediated transactivation of target genes

To mediate transactivation, the GR binds as a homodimer to positive glucocorticoid response elements (GREs) at the promoter region of target genes, increasing in this way their expression [41–43]. The GREs contain the consensus palindromic motif 5’AGAACAnnnTGTTCT3’ (where n represents any nucleotide) to which the GR binds [44]. If the GRE is located in close proximity to the TATA box of a target gene, this leads to direct GR-mediated recruitment of proteins such as Transcription Factor IID (TFIID), which is a key component of the basal transcriptional machinery [45, 46]. If the GRE is distant from the TATA box, other co-activators will bind to the GR and act as bridges to promote transcription [47, 48]. In addition, it has been reported that GR can also recruit chromatin-remodeling coactivators (CRC) that modify the structure of the DNA making it more suitable for transcription initiation. These remodelers are, for example, the histone acetyltransferases (HATs) such as CREB-binding protein (CBP/p300), CBP-associated factor (p/CAF), steroid receptor coactivator-1 (SRC-1) or the ATP-dependent chromatin remodeling factors like SWI/SNF [49–51].
Examples of proteins upregulated by GREs binding-mediated mechanisms are the anti-inflammatory proteins interleukin (IL)-10, annexin A1 and inhibitor of nuclear factor κB (IκB) [52, 53] or the gluconeogenic enzymes tyrosine aminotransferase (TAT), alanine

aminotransferase (ALT), serine dehydrogenase and phosphoenolpyruvate carboxykinase (PEPCK) [7, 54–56].

In another mechanism of transactivation, other proteins such as signal transducers and transcription activators (STATs) have been shown to bind GR. In this case, the GR does not directly interact with DNA, but it is secondarily recruited to the chromatin to promote gene expression. The GR-STAT5 interaction, for example, leads to activation of various genes among which is the hepatic insulin-like growth factor 1 (IGF-1) that is important for postnatal growth [7, 57, 58].

### 1.2.3 Glucocorticoid-mediated transrepression of target genes

Besides the positive transcriptional regulation by GCs, the GR is also known to mediate GCs inhibition of target genes expression via direct interaction as a monomer with negative GREs (nGREs). These GREs are mainly located at the promoter region of genes which are regulated by other transcription factors. The GR competes with them for the DNA-binding site and this leads to an impaired recruitment of proteins of the basal transcriptional machinery and therefore inhibition of the transcription of such genes is called “transrepression” [59]. An example of a gene whose expression is inhibited by GCs through this mechanism is the osteocalcin. Here the GRE overlaps with the TATA box of the gene and therefore the GR binding to it prevents the activity of TFIID [60, 61]. The transcription of other genes like the one of pro-opiomelanocortin (POMC) [62], corticotropin releasing hormone (CRH), bovine prolactin [63] and neural serotonin receptor is also known to be modulated by the binding of monomeric GR to nGREs [59, 64, 65].

Most of the anti-inflammatory effects of GCs however, seem to arise from protein-protein interaction between the GR-hormone complex and other transcription factors. In the nucleus the GR can be tethered to already DNA-bound proteins such as activator protein 1 (AP1), nuclear factor kB (NFkB) or Smad3 [7, 66–68]. In this mechanism, activated GR associates with the p65 subunit of NFkB [66] or the c-Jun subunit of AP1 [67] to diminish the recruitment of members of the basal transcriptional machinery [69] and also co-activators [70]. The GR can also impair p65-mediated HAT activity by recruiting histone deacetylase-2 enzyme to NFkB target genes [71, 72]. Some GR co-activators like the GR interacting protein 1 (GRIP-1), have been shown to have dual activity, functioning also as co-repressors. GRIP1 cooperates with GR to down-regulate AP1 activity through a mechanism that requires both GR and AP1 bound to DNA [73]. Furthermore, the tethered GR can interfere with the phosphorylation of RNA-polymerase II, which is necessary for effective transactivation [67], [74]. All these actions together cooperate to downregulate the activity of pro-inflammatory transcription factors. Examples of proteins whose expression is regulated by GCs through tethering are: collagenase I, stromelysin and other matrix metalloproteinases which are targets of AP1 [67]. Among the targets of NFkB are tumor necrosis factor α (TNFα), IL6, IL8, IL1β, granulocyte monocyte-colony stimulating factor (GM-CSF) [7, 75].

### 1.2.4 Post-transcriptional regulation of mRNA decay by glucocorticoids

Another mechanism for the hormone to inhibit the synthesis of pro-inflammatory cytokines and chemokines consists of the ability of GCs to down-regulate the mRNA stability of these proteins. In particular, GR induces the expression of tristetraprolin (TTP), a protein that is able to bind mRNA containing adenylate/uridylate (AU or ARE)-rich 3' UTR sequences. The TTP-ARE complex recruits specific RNAses that are responsible for the decay of the targeted mRNA. GCs are able in this way to reduce, for example, the half-life of the mRNA of TNFα [76–79]. Other authors have also shown a direct specific binding of the GR to the mRNA of chemokines, for example monocyte chemoattractant protein-1 (MCP-1) in arterial smooth muscle cells, leading to a decrease of its stability [80]. More recently, a novel rapid (30 min) translation-independent mRNA decay pathway called GR-mediated mRNA decay (GMD) has been reported by Cho *et al.* (2015). This requires hormone-bound GR to be loaded onto the 5'UTR of target mRNAs and the recruitment of protein proline-rich nuclear receptor coregulatory 2 (PNRC2) to generate protein upstream frameshift 1 (UPF1) to elicit a rapid mRNA degradation [81].

### 1.2.5 Rapid action of glucocorticoids

#### 1.2.5.1 Three theories

In addition to the above described mechanisms of action of GCs, which require at least 30 minutes to hours, other actions of GCs have been reported to occur very rapidly, within seconds to minutes [55, 82]. Their physiological significance can be attributed to the necessity for the organism to immediately cope with stressful situations. Rapid effects of GCs can also prepare the cells, during the first phase of the inflammatory process, for the later genomic events that lead to inhibition of inflammation [83–85]. Evidence of rapid GCs action has been found in the regulation of hormone secretion, neuronal excitability, carbohydrate metabolism, cell morphology and behavior [86–88] .

Several authors have used different cellular systems or in vivo models to demonstrate and find an explanation for the mechanism of action of the rapid effects of GCs. For instance, GCs have been reported to rapidly inhibit arachidonic acid release and therefore eicosanoids production in adenocarcinoma cells [89]. This was described to occur through downregulation of the recruitment of signaling molecules such as growth factor receptor-bound protein 2 (Grb2) to the membrane epidermal growth factor (EGF) with consequent decrease of phospholipase $A_2$ activity that is required for arachidonic acid liberation [89]. Another example describes high doses of GCs rapidly upregulating the phosphatidylinositol 3-kinase (PI3K)/protein kinase Akt signaling pathway in human vascular endothelial cells [90]. This effect of GCs provides protection of the cardiovascular system from heart attack: stimulation of the PI3K/Akt signaling pathway leads to the activation of the enzyme endothelium nitric oxide synthase (eNOS) with consequent increased synthesis of nitric oxide. Nitric oxide, in turn, has a vasorelaxant activity which is helpful to increase blood flow in case of initial heart infarction [90]. In *in vivo*

experiments, treatment of rats with GCs have been observed to rapidly (within 2 to 7 min) increase the social challenge-induced aggressive behavior of the animals and also their risk assessment during elevated plus-maze and open-field tests. These results in vivo suggest the importance of the hormone in coping with challenging situations [91, 92]. Moreover, GCs have been shown to exert rapid beneficial effects in the treatment of asthma *in vivo* [93] and *in vitro* by inhibiting basal calcium levels in human bronchial epithelial cells [94]. A rapid inhibitory action of GCs on the calcium release was also observed in PC12 cells [95], neuroblastoma cells [96] and muscle cells [97]. Because of the rapidity with which these events occur, their mechanism of action cannot be explained by the classical action of the hormone that requires nuclear transportation of activated GR and modulation of genes expression. It is rather thought that rapid action of GCs is mediated by mechanisms that do not require GR nuclear localization, but arise from the plasma membrane and they are therefore referred to as “non-genomic”. It has been reported that such effects are independent of transcriptional and translational events [55, 89, 98–100]. Over the years, different hypotheses have been postulated for the rapid effects of GCs and several classifications to clarify GCs activity have been proposed [55, 99, 101].

These can be summarized and ascribed to three main theories:

1. Indirect action of GCs via interaction with cellular membranes
2. Direct effects of GCs via interaction with membrane bound GR (mGR)
3. Direct effects of GCs via interaction with the classical cytosolic GR recruited to the plasma membrane

**Indirect action of GCs via interaction with cellular membranes**

Due to their lipophilicity, GCs can easily cross the plasma membrane and enter the cytoplasm. This passage through the phospholipid bilayer increases membrane fluidity and affects the surface topography and viscoelasticity of the plasma membrane [102]. Moreover, it is thought to have implications for ion cycling and ATP consumption at the plasma membrane and also at the mitochondrial membrane [10, 55, 103]. These changes result in modulation of the cell signaling pathways that could account for the rapid effects of GCs. However, very high concentration of hormone (above 10 μM) is necessary for this mechanism to take place [104] and therefore it cannot explain the rapid events that occur employing physiological GCs amount [10].

**Direct effects of GCs via interaction with membrane bound GR (mGR)**

Reports on the existence of a novel receptor, different from the classical cytosolic GR (cGR) began a long time ago, when in 1987 Gametchu reported a GR-like molecule in mouse lymphoma cell membranes [105]. After that time, other studies reported the presence of mGR in amphibian neuronal membranes [106], human leukemic cells [107] and mouse lymphoma cells [108]. In 2004 a mGR, a possible mediator of rapid GCs action, has been described in human monocytes and B lymphocytes [109]. This membrane-bound receptor is most likely encoded by the same gene that codes for the cytosolic GR [110, 111]. It is functional but

however expressed at very low concentration and can only be detected with highly sensitive techniques like Fluorescence Amplification by Sequential Employment of Reagents (FASER) [112]. However, the fact that this protein has still not been well characterized or cloned makes it difficult to exactly understand its function [83]. Furthermore, to confirm the existence of mGR, many studies have been conducted where GCs are fused to a membrane impermeable molecule such as BSA [109, 110]. The complex GCs-BSA can easily dissociate freeing the hormone to pass through the plasma membrane and to bind to the cytosolic GR. In this case, the rapid effects of GCs would be mediated by the cytosolic GR and not by mGR [100].

**Direct effects of GCs via interaction with the classical cytosolic GR recruited to the plasma membrane**

Another model which has been proposed, involves the membrane translocation of the classical GR to mediate the rapid effects of GCs. The mechanisms of the membrane recruitment are still unclear. One of the possibilities for a protein like GR to interact with the plasma membrane is to associate with cytoplasmic proteins that are in turn recruited to the plasma membrane. Hormone-dependent membrane translocation of the human estrogen receptor α (hERα), for example, has been reported in MCF-7 breast cancer cells to occur through its binding to the adaptor protein Shc. The Shc protein carrying the hERα, indeed, interacts in turn with the IGF-1 receptor which is located at the level of the plasma membrane [114]. Another way is to undergo post-translational modifications that change the structure of the receptor and promote its trafficking to the plasma membrane. For example, the covalent attachment of fatty acid such as a palmitic acid to a cysteine (Cys) residue of the receptor characterizes the post-translational protein modification called “palmitoylation”. Cys447, for instance, has been reported to be an important site for the hERα to interact with the plasma membrane through palmitoylation [115, 116]. In addition, it has been shown that this Cys is part of a highly conserved motif in the nuclear receptor superfamily that includes both ER and GR, but also AR and PR (Figure 1.2) [117]. This suggests a possible importance of the homologous of Cys447 in the GR, Cys665, for the membrane localization of GR. This palmitoylation motif is missing in the MR and there is no information on its lipid-modification [118]. Some authors, however, showed that a fraction of MR is localized at the plasma membrane through its interaction with the epidermal growth factor (EGF) receptor [119].

ERα- 445 fvclkssiins
ERβ- 397 ylcvkamiilns
AR- 805 flcmkalllfs
PRB- 818 flcmkvilln
GR- 663 ylcmktllls
Consensus sequence:
Ω ϕ c ϕ ζ ϕ ϕ ϕ ϕ ζ ϕ
n=4 or 5

**Figure 1.2:** Highly conserved motif of the nuclear receptor superfamily

Consensus palmitoylation motif for mouse/human ER, AR, PR and GR. Cys is always in third position surrounded by 9 to 11 highly related amino acids: Ω = aromatic, ϕ=hydrophobic, ζ=hydrophilic [117]

In another mechanism for the rapid action of GCs, the GR was reported to interact with caveolin-1, one of the main components of invaginations of the plasma membrane called lipid rafts or caveolae [113, 120]. However, the association of the GR with caveolin-1 as fundamental for its membrane localization is controversial [52] and Vernocchi *et al.* in 2013 concluded that caveolin-1 modulates the receptor function but it is not required for its membrane localization [110].

Evidence of membrane recruitment of GR was shown for the first time in activated RBL-2H3 mast cells by Oppong *et al.* in 2014. In this study the localization of GR expressed as a GFP fusion was analyzed in a single-cell approach by fluorescence microscopy. The cells were activated on allergen dinitrophenol (DNP)-lipid array produced by dip-pen nanolithography [121] and total internal reflection microscopy (TIRF) was carried out to detect the GR within the small penetration thickness of 100 nm considered as the proximity of the cell membrane. A rapid GC-independent and even stronger hormone-dependent recruitment of GR-GFP was observed within 15 min following contact of the cells with the allergen array (Figure 1.3) [100].

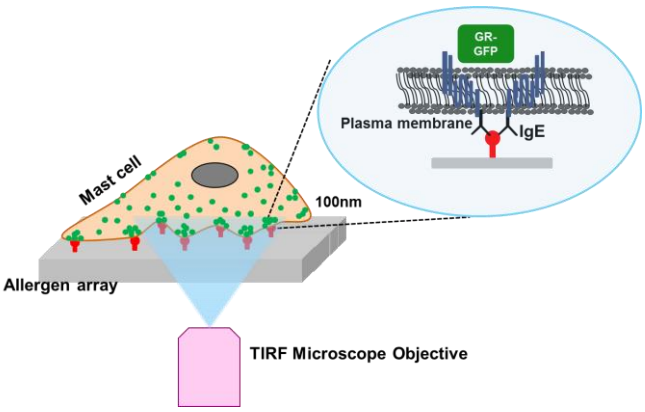


**Figure 1.3:** Scheme of GR recruitment to the plasma membrane

Schematic view of mast cells on the allergen patterns. RBL-2H3 mast cells sensitized with anti-DNP Immunoglobulin E (IgE) are applied on DNP patterns (red). The cells stably express GR-GFP (green) and the recruitment of GR-GFP to DNP patterns is captured by TIRF microscopy in time lapse mode. The light blue oval image is a schematic zoomed view of the 100 nm focus that characterizes the path analyzed by TIRF microscopy.

#### 1.2.5.2 Rapid effects of glucocorticoids on immune cells

GCs have been documented to exert rapid effects on immune cells such as T lymphocytes, basophils and mast cells [100, 122, 123]. Macrophage phagocytosis [124] and neutrophil degranulation [125] are also shown to be rapidly modulated by GCs, while dendritic cells and B lymphocytes are sensitive to the action of the hormones, but the response is not so rapid [83].

T lymphocytes, also called T cells, are activated via crosslinking of the T-cell receptor (TCR) on their surface with an external pathogen (antigen). This occurs through the phagocytosis of the pathogen by antigen presenting cells such as macrophages, dendritic cells or B lymphocytes. The antigen presenting cells digest the antigen and display its fragments on their surface, bound to proteins called “major histocompatibility complex” (MHC). The formed MHC-antigen complex is then recognized by the TCR which binds to it and this triggers T-cell activation.

At the molecular level, the T-cell activation consists of the recruitment to the TCR of signaling molecules such as lymphocyte-specific protein tyrosine kinase (Lck) and Fyn which are phosphorylated. In turn, these kinases phosphorylate other proteins and initiate the signaling cascade that leads to release of inflammatory cytokines and chemokines [126, 127 and reviewed in 128]. It has been described that GCs such as Dex, rapidly affect the function of the Src family tyrosine kinases Lck and Fyn [129]. In particular, within few minutes, Dex can repress the phosphorylation of Lck and Fyn and promote their dissociation from the TCR protein complex [129, 130]. Moreover, the activation of signaling molecules downstream of the TCR, such as protein kinase B (PKB or Akt), PKC and MAPKs, can also be down-regulated by 10 minutes of pre-incubation with Dex [129, 130]. Other authors, on the other hand, reported a rapid GC-mediated increase of tyrosine-phosphorylation of the zeta-chain-associated protein kinase 70 (ZAP-70), a key molecule of the Syk family which is recruited to the TCR upon activation of Lck [131, 132]. The ZAP-70 kinase, in turn, activates LAT and other signaling molecules [133]. The rapid phosphorylation of ZAP-70 by GCs has been reported to be directed to both the inhibitory and activating sites of the kinase with consequent dual response: repression or activation of ZAP-70 [132, 134].

Basophils and mast cells are essential effector cells in allergy, anaphylaxis and autoimmune diseases [135]. It has been reported that pre-treatment of basophils with GCs rapidly (within 30 min) reduces the formation of membrane lipid rafts nanoclusters where the IgE-receptor resides [135], with consequent decreased activity of the cells and therefore reduced inflammation. This is believed to occur through the interaction of GCs with mGR which has been found on the basophil cell membrane and whose activity was not inhibited by the classical GR inhibitor RU486 [135].

## 1.3 Mast cells as a model system to study rapid glucocorticoids action

Mast cells are effector cells of the innate [136] and acquired [137] immune response that play a central role in allergy and inflammation [138] and they are also known to rapidly respond to GCs treatment [122, 123]. Mast cells represent the first line of defense for the organism. They have been found in tissues and serosal cavities which are more exposed to the environment and external pathogens, for example the skin, the respiratory or intestinal tract mucosa or in close vicinity of blood vessels and nerves [139–142]. They originate in the bone marrow from pluripotent hematopoietic precursors and they subsequently migrate through the blood stream or the lymphatic system to various tissues where they become matured under the influence of specific growth factors [137, 143–147] such as the stem cell factor (SCF or c-Kit ligand) and IL-3 [147–151]. A peculiarity of mast cells is the expression of the membrane tyrosine kinase receptor (c-Kit) from the progenitor stage throughout their entire survival. This makes mast cells always responsive to SCF [147]. In other hematopoietic progenitors, the c-Kit receptor undergoes down-regulation during the differentiation steps and it is not expressed anymore at the mature stages [147]. SCF is not only important for proliferation and differentiation of mast cells, but it also acts as chemotactic factor that draws mast cells to their final niche [152–156]. IL-3 is a cytokine that has similar functions to the SCF and it is an essential mast cells survival factor [157]. IL-4, IL-9, IL-10 and nerve growth factor (NGF) are other factors known to contribute to mast cells growth and differentiation [156].

There are two phenotypes of mast cells depending on the site where they reside and their secretory granules content: connective tissue-type or mucosal type mast cells [141, 158, 159]. This heterogeneity arises from the different composition of growth factors that characterizes the microenvironment of the tissues where the premature cells differentiate in mature mast cells [156]. Connective tissue mast cells, also called serosal mast cells, are mostly found in the skin, peritoneal cavity [160] and in the connective tissue of various organs [161]. They are characterized by high content of heparan sulfate proteoglycans, histamine, chymases (mMCP-4,-5), tryptases (mMCP-6,-7) and mast cell carboxypeptidase A [162]. Mucosal type mast cells are smaller and reside mainly in the intestinal lamina propria and the mucosa of the respiratory system [147]. They contain less granules with chondroitin sulfate proteoglycan, histamine and chymases (mMCP-1,-2 [162, 163]), but not tryptases [147]. The two types of cells do not differ only in their proteases content (chymases and tryptases) and in size, but also in their function. For example, mucosal mast cells has been reported to considerably expand during T cell-mediated immune response to intestinal parasitosis [164, 165], while connective tissue mast cells manifested little or no T cell-influence on their proliferation [166], suggesting different activity. Bone marrow derived mast cells (BMMCs) are considered immature mast cells that can, in principle, differentiate into both phenotypes [144], but they remind more connective tissue mast cells in their protease content [167].

### 1.3.1 Activation of mast cells

Mast cells not only express on their surface the c-Kit receptor, but a lot of other receptors such as the high-affinity IgE receptor (FcεRI) or the toll-like receptor (TLR) which make them possible to respond to different stimuli for activation. For example, mast cells are well known to play a central role in type I hypersensitivity and they also rapidly react against parasites or other pathogens (bacteria, viruses or fungi) by releasing inflammatory mediators such as their granules content, arachidonic acid and its metabolites, cytokines and chemokines [168–174]. Mast cells activation, moreover, leads to increased capillary permeability (histamine effect) and recruitment of other immune cells to the site of the infection; for example eosinophils, neutrophils, natural killers (NKs) or T cells which are responsible for hosts detection and elimination [172]. Among the variety of surface receptors, the FcεRI receptor has been the most investigated over the past years [137, 175]. In mice and rats, this receptor is not only expressed on the membrane of mast cells, but also of basophils [138, 176, 177]. It belongs to the protein family of immunoglobulin Fc-receptors, and consists of a tetrameric structure composed by an α-chain, carrying an extracellular domain important to bind the Fc fragment of the IgE, a transmembrane β-subunit and two γ-chains linked via disulfide bond. These latter ß- and γ-chains have a transmembrane portion and a cytoplasmic portion which contains immunoreceptor tyrosine-based activation motifs (ITAMs) responsible for the signal transduction and propagation [177–180]. Following the first contact with an allergen, B-lymphocytes produce specific IgE which bind to the FcεRI receptors on the mast cell surface. At the second exposure to the same antigen, the crosslink of two IgE-FcεRI complex by the allergen occurs, which activates the mast cell and triggers the inflammatory reaction. This consists of a cascade of phosphorylation of cytoplasmic protein kinases and calcium mobilization [171]. Other surface receptors such as the Kit receptor have been described to cross-talk with the FcεRI pathway enhancing its activity [169, 174, 181, 182].

#### 1.3.1.1 Degranulation pathway

Degranulation is the first event following IgE receptor crosslinking and it takes place within 5 - 30 min [169, 172]. In this process, preformed molecules stored in mast cell cytoplasmic vesicles are released in the surrounding environment [147, 183]. These mediators include biogenic amines like histamine [184, 185], serotonin [186] and dopamine [187–189]. Lysosomal enzymes are also stored and released upon mast cell activation. ß-hexosaminidase is the best defined lysosomal enzyme due to its frequent use as an indicator of mast cells degranulation in *in vitro* studies [190]. Some cytokines like TNFα are pre-formed and stored in mast cell granules in order to be rapidly released when needed [190–193]. A number of proteases (chymases, tryptases and mast cell carboxypeptidase A) are stored in mast cell as functioning enzymes bound to heparin or chondroitin proteoglycans [194–199].

The first molecules involved in the mast cells activation pathway mediated by FcεRI crosslinking are the Src family tyrosine kinases Lyn and Fyn. These proteins are recruited to the IgE receptor where they get activated [200]. At this location, active **Lyn** phosphorylates the

tyrosine residues of the ITAMs present in the ß and γ subunits of the receptor [175, 195, 201] and this permits the subsequent recruitment and activation of another initiator of the signaling cascade, the spleen tyrosine kinase (**Syk**) [168, 173, 195, 202]. Syk, in turn, phosphorylates the transmembrane molecular adaptors "linker for activation of T cells" **LAT1** and "LAT-related transmembrane adaptor" **LAT2** [202] which then recruit other cytosolic adaptor proteins to form a multi-molecular signaling complex [174, 203, 204]. Particularly important for the degranulation pathway is the recruitment to the LAT complex of the phospholipase C gamma (**PLCγ**) [169, 205, 206]. This protein directly binds to LAT through its SH2 domain and such interaction is stabilized by SLP76 [206, 207]. Once activated, this enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate (**PIP2**) to produce diacylglycerol (**DAG**) and inositol trisphosphate (**$IP_3$**) [169, 208, 209]. DAG promotes protein kinase C (**PKC**) phosphorylation which has been reported to be involved in cytoskeleton reorganization and secretory granules fusion with the plasma membrane in a calcium independent manner [169, 203, 209]. $IP_3$, on the other hand, generates a massive and prolonged increase of cytoplasmic **calcium** ($Ca^{2+}$) by binding $IP_3$ receptors on the membrane of the ER [169, 209, 210]. The spilling out of intracellular $Ca^{2+}$ from its reservoirs stimulates the recall of extracellular $Ca^{2+}$ [174, 211–215]. The strong $Ca^{2+}$ influx leads to cytoskeletal filamentous actin de-polymerization and disassembly and this facilitates the secretory granules to move to the plasma membrane [216–221].

The protein kinase **Fyn** has also been described to be involved in the degranulation pathway. Once activated, it phosphorylates, together with Syk, the adaptor protein **Gab2** [200, 222, 223] which forms a complex with Grb2 on activated LAT [204]. Activation of Gab2 allows the recruitment of the protein phosphatidylinositide 3-kinase (**PI3K**). PI3K phosphorylates PIP2 at the plasma membrane enabling its conversion to phosphatidylinositol 3,4,5-trisphosphate (**PIP3**) and creating in this way anchor sites for other proteins such as the Bruton's tyrosine kinase (**Btk**) [174, 195, 224]. Btk, PI3K and Gab2 can activate guanosine triphosphatases (GTPases) of the **Rho** family such as RhoA and Rac; this helps microtubule formation, cytoskeleton reorganization and degranulation in a calcium independent manner [220, 225]. PI3K-dependent activation of Btk leads to phosphorylation of **Grb2** which is in complex with LAT1 and LAT2 and this promotes the enhancement and preservation of the PLCγ pathway which leads to calcium influx necessary for degranulation [182, 226]. Furthermore, it has been reported that PI3K can also indirectly interact with **PKC** via PI3K-dependent kinase 1 (**PDK1**) facilitating in this way its function in mediating exocytosis [200, 223, 227]. A simplified scheme of the above described mast cells pathway is shown in Figure 1.4.

#### 1.3.1.2 Arachidonic acid pathway

Release of arachidonic acid and synthesis of its metabolites (eicosanoids) upon FcεRI-mediated mast cells stimulation is the event that, in terms of time, immediately follows degranulation [169, 172]. **Eicosanoids** (prostaglandins, thromboxane and leukotrienes) are lipid mediators which contribute to the increase of vascular permeability, bronchoconstriction, vasodilation and chemo-kinesis during inflammation [228]. They are derived from the

enzymatic action of proteins such as 5-lipoxygenase or cyclooxygenase (COX) on **arachidonic acid** [228, 229]. The latter is a polyunsaturated fatty acid that is usually found in the phospholipids that constitute the plasma membrane and its liberation is catalyzed by the hydrolyzing action of the cytoplasmic enzyme phospholipase $A_2$ (**$PLA_2$**) [229, 230]. As shown in Figure 1.4, the increase in intracellular **calcium** is important for the interaction of $PLA_2$ with the plasma membrane necessary for the liberation of arachidonic acid [168]. $PLA_2$ can also be activated by mitogen-activated protein kinases (**MAPKs**), in particular by the extracellular signal-regulated kinase (**Erk**) [168, 231–233].

#### 1.3.1.3 Cytokines and chemokines pathway

The last and most time consuming event that occurs hours following antigen stimulation of mast cells is the synthesis of cytokines and chemokines [169, 172, 191] which act as inflammatory mediators. These are, for example, the granulocyte monocyte-colony stimulating factor (GM-CSF), TNFα, the interleukins IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13, chemokines (chemotactic cytokines) such as CCL2, CCL3, CCL5, and CXCL8, and growth factors like SCF [174, 234–238]. The signaling cascade that leads to cytokine gene expression is shown in Figure 1.4 and it is common to the degranulation pathway described in paragraph 1.3.1.1 until the formation of the complex between LAT and the adaptor proteins. This complex formation is necessary to promote the conversion of the GTPase **Ras** from its GDP-bound inactive form to the GTP-bound active one [239–242]. Once activated, Ras binds to **Raf** that, in turn, regulates the activation of **MEK** and the MAPKs **Erk1/2**, c-Jun N-terminal kinase (**JNK**), and **p38** [206, 232, 243–247]. Erk, JNK and p38 are the downstream molecules of this signaling pathway that activate **transcription factors** (TFs) such as members of the activation protein 1 (AP1) family, c-Fos and c-Jun [248], but also nuclear factor-κB (NFκB) [249, 250] and nuclear factor of activated T cells (NFAT) [251]. Active TFs move to the nucleus where they promote the expression of specific cytokine-related genes [173, 182]. Since the activation of TFs of the AP1 family or the NFkB can also occur via PKC phosphorylation [252] and because NFAT stimulation has been shown to be calcium dependent [203, 253, 254], the PLCγ-pathway can be considered important not only for degranulation, but also for the regulation of cytokines and chemokines production in mast cells (Figure 1.4) [182]. In addition to the Lyn pathway, the **Fyn-Gab2-PI3K** cascade leads to TFs activation and cytokines and chemokines synthesis by phosphorylating the protein **Akt** via **PDK1** (Figure 1.4) [255].

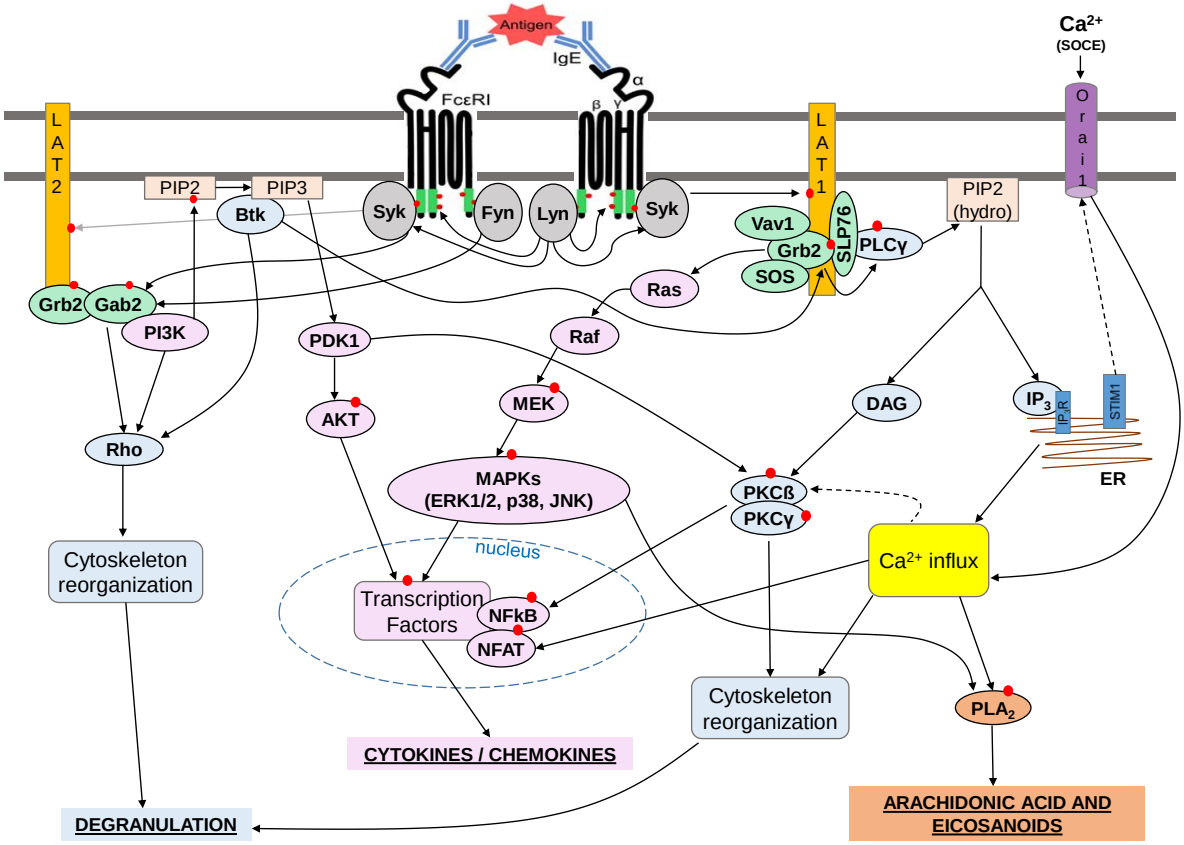


**Figure 1.4:** Simplified scheme of mast cells FcεRI-mediated signaling pathway

Upon binding of the antigen to two FcεRI receptors, Lyn and Fyn protein kinases are recruited to the receptors and activated. Active Lyn phosphorylates the ITAMs of the ß- and γ-chains of the receptor and this leads to recruitment and activation of Syk which then activates LAT1 and LAT2 that attract Grb2, Gab2, SLP76, Vav1 and SOS molecular adaptors to form a complex. Two pathways start from this complex: one via Ras and Raf brings to phosphorylation of MEK and MAPKs (Erk1/2, p38 and JNK) with consequent activation of transcription factors and cytokines and chemokines production; the other one goes through activation of PLCγ and results in the synthesis of DAG and $IP_3$ which are respectively involved in regulation of PKC and calcium signal activation. PKC is active in cytoskeleton reorganization that is necessary for degranulation, but it also activates transcription factors important for cytokines and chemokines release. On the other hand, the increase of calcium influx from endoplasmic reticulum (ER) or extracellular compartment via "store-operated calcium entry" (SOCE), brings to degranulation, lipid mediators release mediated by activation of $PLA_2$ or cytokines and chemokines production due to activation of NFAT. $PLA_2$ can also be activated by MAPKs. In parallel, Fyn and Syk phosphorylate Gab2 that recruits and activates PI3K with subsequent conversion of PIP2 to PIP3 and formation of anchor sites for Btk that enhances and maintains the PLCγ pathway. PI3K, Btk and Gab2 can also contribute to degranulation by activating Rho family proteins involved in cytoskeleton reorganization. PI3K, finally, via PIP3 and PDK1, phosphorylates Akt that, in turn, activates transcription factors and therefore leads to cytokines and chemokines gene expression. Adapted from [225] and [169]. Abbreviations: ITAMs, immunoreceptor tyrosine-based activation motifs; Syk, spleen tyrosine kinase; LAT1, linker for activation of T cells; LAT2, LAT-related transmembrane adaptor; Grb2, growth factor receptor-bound protein 2; Gab2, Grb2-associated binder-like protein 2; SLP76, Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa; SOS, son-of-sevenless; Ras, rat sarcoma; Raf, rapidly accelerated fibrosarcoma; MEK, mitogen-activated protein kinase kinase (or MAPKK); MAPKs, mitogen-activated protein kinases; PLCγ, phospholipase Cγ; DAG, diacylglycerol; $IP_3$, inositol trisphosphate; PKC, protein kinase C; $PLA_2$, phospholipase $A_2$; NFAT, nuclear factor of activation of Tcells; PI3K, phosphatidylinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Btk, Bruton's tyrosine kinase; PDK1, PI3K-dependent kinase 1; Akt or protein kinase B.

### 1.3.2 Effects of glucocorticoids on mast cells function

Long term GCs administration has been described to down-regulate the antigen-mediated mast cells activation [238, 256, 257]. For example, Erk1/2 and $PLA_2$ phosphorylation in mast cells were repressed by GCs through disruption of the interaction between Raf1 and HSP90 which is necessary to traffic Raf1 to the plasma membrane where it is activated [257–259]. The phosphorylation of Erk1/2 was also shown to be inhibited by GCs at 8 h in BMMCs [260] and at 16 h in RBL-2H3 [261]. The latter was an indirect function of the hormone and was mediated by increased expression and decreased degradation of the protein phosphatase MAP kinase phosphatase 1 (MKP1 or DUSP1).
Activation of JNK [256] and p38 [260] were also demonstrated to be down-regulated by prolonged treatment with GCs (6h JNK, 4h p38) in mast cells.

Additionally, the anti-inflammatory effect of GCs in mast cells has been reported to be mediated by their up-regulation of the expression of the MAPKs-inhibitory adaptor molecules Src-like adaptor protein 1 (SLAP1) and downstream of tyrosine kinase 1 (Dok1) [262–264]. These proteins affect upstream events of the signaling cascade in mast cells response. For example, SLAP1 inhibits LAT, PLCγ and $Ca^{2+}$ influx, leading to a decrease in degranulation, arachidonic acid and cytokines release [263]. Dok1 has also been reported to recruit Ras GTPase-activating protein (RasGAP) which inactivates Ras and its signaling pathway [262], [265].

On the other hand, there are only a few reports about the effects of short term GCs administration in mast cells. For example, experiments carried out in guinea pigs by Zhou *et al.* (2003) revealed that the GC budesonide significantly and rapidly (within 10 min) decreased lung resistance and dynamic lung compliance which are parameters indicative for the severity of asthma [93]. At the cellular level, Zhou *et al.* (2008) also showed that budesonide inhibited, within 10 min, the degranulation of lung tissue mast cells in the guinea pigs with allergic asthma. In addition, they performed *in vitro* experiments using RBL-2H3 mast cells and demonstrated that the GC corticosterone could, within 15 min, inhibit the release of histamine from these cells. This effect was independent of the genomic action of the classical GR since it was insensitive to the RU486 inhibitor and the protein synthesis inhibitor cycloheximide (CHX) [123]. Similar results from the study of Zhou et al. (2008) were also obtained by Liu *et al.* (2007) in rat peritoneal mast cells [122]. In another recent study, Oppong et al. (2014) reported a rapid (5 - 9 min) Dex-dependent up-regulation of phosphorylation of Erk1/2 in RBL-2H3 mast cells [100].

Taken together, these studies show how GCs use multiple mechanisms to exert their effects on mast cell function.

## 1.4 Aim

GCs have been reported to rapidly inhibit mast cells function, but the mechanism of action is still poorly understood. A knowledge of how GCs rapidly suppress allergy and inflammation mediated by these cells will aid in the development of new drugs with a better benefit-risk ratio. The aim of this research work was to study the rapid action of GCs in mast cells and investigate the involvement of the GR as a mediator of the rapid effects. This was done by using knock-out GR primary mast cells. Rapid mechanisms of action of GCs have been postulated to occur through membrane localization of the GR. A further goal of the present project was, therefore, to better characterize the mechanisms of the membrane localization of the GR. To determine this, mast cells transfected with a fluorescent mutant GR were immobilized on allergen arrays and the localization of the GR at the plasma membrane was analyzed by fluorescence microscopy. In addition, the dynamics of the localization of the GR at the plasma membrane of mast cells was also investigated by single molecule tracking.

# 2 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals

All the chemicals used for the project are listed in the following Table 2.1

**Table 2.1:** Chemicals and their suppliers

| Chemical | Supplier |
|---|---|
| 4-Nitrophenyl N-acetyl-β-D-glucosaminide (pNAG) | Sigma-Aldrich Chemie, Steinheim |
| 5X Green GoTaq reaction buffer | Promega, Mannheim |
| Adenosintriphosphat (ATP) | Sigma-Aldrich Chemie, Steinheim |
| Ammonium chloride ($NH_4Cl$) | Roth, Karlsruhe |
| Ammonium persulfate (APS) | Roth, Karlsruhe |
| Bovine serum albumin (BSA) powder | GE Healthcare Life Science, USA |
| Bromophenol blue | BioRad, Heidelberg |
| Calcium chloride ($CaCl_2$) | Roth, Karlsruhe |
| Citric acid | Roth, Karlsruhe |
| Coelenterazin substrate 1mM | Biosynth, Gstaat, Schweiz |
| Copper (II) sulfate ($CuSO_4$) | Jena Bioscience, Germany |
| Desoxyribonucleoside triphosphate (dNTP) | Roche, Mannheim |
| Disodium hydrogen phosphate ($Na_2HPO_4$) | Roth, Karlsruhe |
| Dithiothreitol (DTT) | Roth, Karlsruhe |
| DNP-azide | Jena Bioscience, Germany |
| Ethanol (EtOH), extra pure | VWR Chemicals, France |
| Ethidium bromide | Roth, Karlsruhe |
| Ethylene diamine tetra acetate (EDTA) | Roth, Karlsruhe |

| Chemical | Supplier |
|---|---|
| Ethylene glycol tetraacetic acid (EGTA) | Roth, Karlsruhe |
| Glucose | Roth, Karlsruhe |
| Glycerol | Roth, Karlsruhe |
| Glycine | Roth, Karlsruhe |
| Glycylglycin | Sigma-Aldrich Chemie, Steinheim |
| GoTaq polymerase 5U/µl | Promega, Mannheim |
| HEPES | Roth, Karlsruhe |
| Isopropanol | Roth, Karlsruhe |
| Magnesium chloride ($MgCl_2$) | Roth, Karlsruhe |
| Magnesium sulfate ($MgSO_4$) | Roth, Karlsruhe |
| Methanol (MetOH) | Roth, Karlsruhe |
| Milk powder | Saliter, Obergünzburg |
| PageRuler™ Prestained Protein Ladder | Thermo Fisher Scientific, USA |
| Passive lysis buffer (5X) | Promega, Mannheim |
| PeqGOLD Universal Agarose | PeqLab Biotechnologie GmbH, Erlangen |
| Potassium chloride (KCl) | Roth, Karlsruhe |
| Potassium hydrogen carbonate ($KHCO_3$) | Roth, Karlsruhe |
| Potassium phosphate dibasic ($K_2HPO_4$) | Roth, Karlsruhe |
| Potassium phosphate monobasic ($KH_2PO_4$) | Roth, Karlsruhe |
| Proteinase K | Roth, Karlsruhe |
| Sodium acetate | Roth, Karlsruhe |
| Sodium ascorbate | Jena Bioscience, Germany |
| Sodium chloride (NaCl) | Roth, Karlsruhe |
| Sodium dodecyl sulfate (SDS) | Roth, Karlsruhe |
| Sodium hydroxide (NaOH) | Roth, Karlsruhe |
| Tetramethylethylenediamine (TEMED) | Roth, Karlsruhe |
| Tris-base | Roth, Karlsruhe |
| Tris-hydrochloride (tris-HCl) | Roth, Karlsruhe |
| Triton X-100 | Roth, Karlsruhe |

| Chemical | Supplier |
|---|---|
| Tween® 20 | Roth, Karlsruhe |
| Western Blotting Substrate (ECL) | BioRad, Heidelberg |
| β-mercaptoethanol | Sigma-Aldrich Chemie, Steinheim |

### 2.1.2 Equipment

The equipment necessary for this project is listed in Table 2.2

**Table 2.2:** Equipment and suppliers

| Description | Supplier |
|---|---|
| Andor Revolution® XD spinning disk laser scanning microscope | BFi OPTiLAS, München |
| Biological safety cabinet (EN12469) | Thermo Fisher Scientific, USA |
| Branson Sonifier Cell Disruptor B15 | G. Heinemann Ultraschall- und Labortechnik Schwäbisch Gmünd |
| Cast-It M caster | Peqlab Biotechnologie GmbH, Erlangen |
| ChemiDoc Touch Imager | BioRad, Heidelberg |
| ELx808IU Ultra Microplate reader | BioTek Instruments, Germany |
| Eppendorf Centrifuge 5804 | Eppendorf AG, Hamburg |
| Eppendorf Microcentrifuge 5417R | Eppendorf AG, Hamburg |
| Eppendorf Thermomixer 5436 | Eppendorf AG, Hamburg |
| Gene Pulser® Transfection Apparatus | BioRad, Heidelberg |
| Heraus Biofuge Pico | Kendro, Hanau |
| Image Lab™ Software | BioRad, Heidelberg |
| ImageJ | National Institute of Health, USA |
| Immobilon®-P membrane (PVDF) | Merck Chemicals GmbH, Darmstadt |
| Inverted microscope Axiovert 200 | Carl Zeiss, Germany |
| Inverted microscope TE2000 | Nikon, Germany |
| Leica DMIL | Leica Microsystems, Wetzlar |
| NLP 2000 instrument | Nanoink Inc., USA |
| PerfectBlue Duel Gel System Twin M | Peqlab Biotechnologie GmbH, Erlangen |
| PerfectBlue Gel System | Peqlab Biotechnologie GmbH, Erlangen |

| Description | Supplier |
|---|---|
| Sterile cell cuture $CO_2$ incubator | Labotect, Göttingen |
| Trans®-Blot Cell | BioRad, Heidelberg |
| Transilluminator | Peqlab Biotechnologie GmbH, Erlangen |
| Victor™ Light 1420 Luminescence counter | Perkin Elmer precisely, USA |

### 2.1.3 Cell culture

All cell culture materials used for the project are listed in Table 2.3
The different types of cells used in this study are recorded in Table 2.4

**Table 2.3:** Cell culture materials

| Description | Supplier |
|---|---|
| 0.2 cm electroporation cuvette | BioRad, Heidelberg |
| 1X Dulbecco's Phosphate-Buffered Saline (PBS) | GIBCO®, Thermo Fisher Scientific, USA |
| 70 µm falcon cell strainer | Corning®, Kaiserslautern |
| Alexa647 labeling IgE Kit | Molecular Probes®, Thermo Fisher Scientific, USA |
| Cell culture dishes, flasks and multi-well plates | Greiner Bio-One GmbH, Frickenhausen |
| Cycloheximide | Sigma-Aldrich Chemie, Steinheim |
| Dexamethasone | Sigma-Aldrich Chemie, Steinheim |
| DNP-BSA (albumin from bovine serum, 2,4-dinitrophenylated) | Molecular Probes®, Thermo Fisher Scientific, USA |
| Dulbecco's Modified Eagle Medium (DMEM) | GIBCO®, Thermo Fisher Scientific, USA |
| FBS from south America origin | GIBCO®, Thermo Fisher Scientific, USA |
| Fetal bovine serum (FBS) | Sigma-Aldrich Chemie, Steinheim |
| Iscove's Modified Dulbecco's Medium (IMDM) | GIBCO®, Thermo Fisher Scientific, USA |
| L-glutamine | GIBCO®, Thermo Fisher Scientific, USA |
| Murine Interleukin-3 (IL-3), recombinant | PreproTech, Rocky Hill, NJ |
| Murine monoclonal anti-dinitrophenyl IgE (clone SPE-7) | Sigma-Aldrich Chemie, Steinheim |
| Murine stem cell factor (SCF) | Kindly provided by Stassen M. [266] |

| Description | Supplier |
|---|---|
| Neomycin G418 | Sigma-Aldrich Chemie, Steinheim |
| Nunc™Lab-Tek™Chambered Coverglass | Thermo Fisher Scientific, USA |
| Penicillin/Streptomycin 10,000 U/ml | GIBCO®, Thermo Fisher Scientific, USA |
| PromoFectin | PromoCell GmbH, Heidelberg |
| Pyruvate | GIBCO®, Thermo Fisher Scientific, USA |
| RU486 | Sigma-Aldrich Chemie, Steinheim |
| Trypsin-EDTA | GIBCO®, Thermo Fisher Scientific, USA |

**Table 2.4:** Cell types used for the project and their culture medium

| Cell type | Description | Medium |
|---|---|---|
| RBL-2H3 | Rat basophilic leukemia cell line | DMEM + 15% FBS |
| COS-7 | Monkey (Cercopithecus aethiops) kidney fibroblast-like cell line | DMEM + 10% FBS |
| BMMCs | Bone marrow derived mast cells (murine primary cells) | IMDM + 10% FBS, 2 mM L-glu, 1 mM pyr, 1% Pen/Strep, 100 ng/ml SCF, 5-10 ng/ml IL-3, 50 µM 2-mercaptoethanol |
| PCMCs | Peritoneal cell-derived mast cells (murine primary cells) | |

### 2.1.4 Antibodies

Primary and secondary antibodies that were used for Western blot analysis are summarized in the following Table 2.5.

**Table 2.5:** Antibodies for Western blot analysis

| Primary Antibody | Supplier | Use |
|---|---|---|
| Erk1 (K-23) sc-94<br>rabbit polyclonal IgG, 200 µg/ml | Santa Cruz, Heidelberg | 1:1000 in 5% milk<br>3 h at RT or overnight at 4°C |
| GR (FiGR) sc-12763<br>mouse monoclonal $IgG_{2b}$ 200 µg/ml | Santa Cruz, Heidelberg | 1:1000 in 5% milk<br>overnight at 4°C |
| NCL-GCR<br>lyophilized mouse monoclonal antibody | Leica Biosystems, Nussloch | 1:500 in 5% milk<br>overnight at 4°C |

| Primary Antibody | Supplier | Use |
|---|---|---|
| p38α (C-20) sc-535<br>rabbit polyclonal IgG, 200 µg/ml | Santa Cruz, Heidelberg | 1:1000 in 5% milk overnight at 4°C |
| Phospho-p38 MAPK (T180/Y182)<br>rabbit mAb | Cell Signaling Technology, USA | 1:1000 in 5% BSA overnight at 4°C |
| Phospho-p44/42 MAPK (T202/Y204)<br>rabbit mAb | Cell Signaling Technology, USA | 1:2000 in 5% BSA 3 h at RT or overnight at 4°C |
| Phospho-SAP/JNK (T183/Y185)<br>rabbit mAb | Cell Signaling Technology, USA | 1:1000 in 5% BSA overnight at 4°C |
| ß-Actin (C4) sc-47778<br>mouse monoclonal $IgG_1$ 200 µg/ml | Santa Cruz, Heidelberg | 1:1000 in 5% milk<br>3 h at RT or overnight at 4°C |
| **Secondary Antibody** | **Supplier** | **Use** |
| Goat-anti-mouse IgG (H+L) HRP conjugate | Advansta, USA | 1:10,000 in 5% milk or BSA 45 min at RT |
| Goat-anti-rabbit IgG (H+L) HRP conjugate | Advansta, USA | 1:10,000 in 5% milk or BSA 45 min at RT |

### 2.1.5 Oligonucleotides for genotyping

All the primers used for genotyping were purchased from Metabion, Martinsried and are listed in the following Table 2.6. Mcpt5-Cre primers refer to Scholten *et al.* [267], while GRflox primers refer to Prof. Dr. Jan Tuckermann (university of Ulm) guidelines.

**Table 2.6:** Oligonucleotides for genotyping

In case of the Mcpt5-Cre typing, a third primer (Primer 3) was used to amplify together with the upstream primer (Primer 1) a product representing the WT Mcpt5 locus. This PCR product, which should always be present, served as an internal control.

| Name | Sequence | Products of the PCR |
|---|---|---|
| Primer 1: Mcpt5-CreUP | 5'ACAGTGGTATTCCCGGGGAGTGT | WT: 224 bp (always)<br>Mcpt5-Cre TG: 554 bp |
| Primer 2: Mcpt5-CreDO | 5'GTCAGTGCGTTCAAAGGCCA | |
| Primer 3: Mcpt5-Ex1-DO3 | 5'TGAGAAGGGCTATGAGTCCCA | |
| Primer 1: GR1 | 5'GGCATGCACATTACTGGCGTTCT | WT: 225 bp<br>GR loxP: 275 bp<br>GR null: 360 bp |
| Primer 2: GR4 | 5'GTGTAGCAGCCAGCTTACAGGA | |
| Primer 3: GR8 | 5'CCTTCTCATTCCATGTCAGCATGT | |

### 2.1.6 Plasmids

All the plasmids used for this study are reported in Table 2.7

**Table 2.7**: Plasmids and their sources

| Plasmid | Source |
|---|---|
| GFP | Obtained from Clontech (Göttingen, Germany) |
| mEos2-GR | Kindly provided by Gebhardt, J.C.M. [268] |
| pk7 GR-GFP | The GR was cloned by Oppong E. [100] into the Xbal site of pk7-GFP (kindly provided by Macara, I.G. and Charlottesville, VA) such that the stop codon of GR was deleted and the coding sequence continued in frame with that of the GFP. |
| pk7 GR-GFP MutC665A | The construct was obtained by point mutating the plasmid pk7 GR-GFP at position Cys 665 by Alanine (Bohem, M.) |
| psV2neo | |
| pGL3 MMTV-Luc | Encodes the Photinus pyralis (firefly)-luciferase gene under the control of the long terminal repeat region (-241 to -137) of the mouse mammary tumor virus (MMTV), which was cloned as a Bam HI / Bg1 III fragment into the pGL3 basic vector [269]. |
| pTK Renilla Luc | Promega, Mannheim (Renilla reniformis luciferase) |

## 2.2 Methods

### 2.2.1 Mice

All animal experiments were performed according to European and German statutory regulations.

#### 2.2.1.1 Mice strain

The mice used in this study were on the C57BL/6 background, purchased from Harlan Winkelmann, Borchen in 1995 and crossed exclusively to C57BL/6 mice. Beside wild-type C57BL/6 mice, a new line called “B6, Mcpt5-Cre GRflox, alf Cre” was generated to obtain specific peritoneal mast cells knock-out of GR.

#### 2.2.1.2 Cre/loxP system to generate specific tissue knock-out of GR

To generate a specific tissue knock-out of GR, the Cre/loxP system was used. “Mcpt5-Cre” mice (CreTG/$GR^{wt/wt}$) were purchased from Prof. Dr. Axel Roers (university of Dresden [267]) and “GRflox, alf Cre” ($GR^{flox/flox}$) mice from Prof. Dr. Jan Tuckermann (university of Ulm). In the first animal line, the transgenic mice were expressing Cre-recombinase under the control of the promoter Mcpt5 which is specifically active in mature connective tissue mast cells like peritoneal mast cells. The second mouse line carried the GR gene flanked by two loxP sites

which are recognized and cut by Cre-recombinase once that they are simultaneously expressed in the same cell. To obtain mast cells expressing both Cre-recombinase and loxP-flanked GR, further breeding of the animals was performed in the animal facility of the Institute of Toxicology and Genetics under specific pathogen free (SPF) conditions following the scheme in Figure 2.1. Note that in transgenic mice (CreTG), it is not possible to distinguish between heterozygous and homozygous mice by PCR. Therefore, transgenic mice were never crossed to each other.

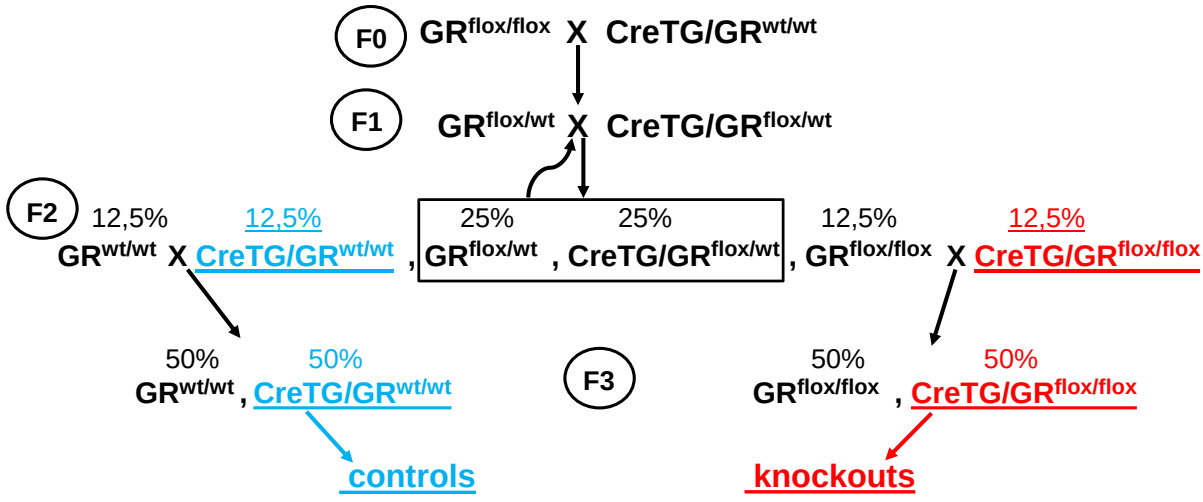


**Figure 2.1:** Scheme of mice crossing to obtain GR knock-out mast cells.

F0 mice were purchased and crossed to obtain the F1 generation. Further mating of F1 mice as shown in this scheme led to obtain 12.5% of control and 12.5% of knock-out mice (F2). To increase this percentage to 50% another breeding was carried out to generate a control line (light blue) and a knock-out line (red). Mice of the same age coming from the two lines (F3) or the F2 generation were then used for experiments.

### 2.2.2 Genotyping

Genotyping was carried out from mice tails and cell pellet using the primers listed in Table 2.6 and the following method.

#### 2.2.2.1 Isolation of genomic DNA

A small piece of tail or cell pellet obtained from circa $10^5$ cells was incubated in 200 µl of lysis buffer (Table 2.8) overnight at 55°C while shaking using an Eppendorf Thermomixer 5436 (Eppendorf AG, Hamburg). Two hundred µl (200 µl) of Isopropanol were then added and after vortexing for few seconds, the precipitated DNA was pelleted by centrifugation at 14,000 rpm for 5-10 min. Supernatant was discarded and the DNA pellet allowed to air dry. The DNA was then dissolved in TE buffer (Table 2.8) by incubating at 37°C in heating block (Eppendorf Thermomixer 5436) for at least 2 h while shaking.

**Table 2.8:** Buffers for DNA isolation

| | |
|---|---|
| **Lysis buffer** | 100 mM tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/ml Proteinase K |
| **TE buffer** | 10 mM tris-HCl, 0.1 mM EDTA pH 8 |

### 2.2.2.2 Polymerase chain reaction (PCR)

One µl of isolated DNA was added in a PCR tube to 19 µl of Master Mix prepared as described in Table 2.9 and then the PCR program shown in Table 2.9 was run.

**Table 2.9:** Genotyping PCR protocol

| **Name** | **PCR- Master Mix (1X)** | **PCR program** |
|---|---|---|
| Mcpt5-Cre | 13.8 µl $dH_2O$<br>5 µl 5X Green GoTaq reaction buffer<br>2 µl 2 mM dNTPs<br>1 µl 10 pmol/µl Primer 1<br>1 µl 10 pmol/µl Primer 2<br>1 µl 10 pmol/µl Primer 3<br>0.2 µl GoTaq Polymerase 5 U/µl<br>Take 19 µl of mix and add 1 µl of DNA | 95°C 5 min<br>95°C 45 sec<br>57°C 1 min } 29x<br>72°C 45 sec<br>72°C 7 min<br>10°C Forever |
| GRflox | | 94°C 2 min<br>94°C 20 sec<br>55°C 20 sec } 35x<br>72°C 1.30 min<br>72°C 7 min<br>15°C Forever |
| **TAE buffer** | 40 mM tris-base pH 7.2, 20 mM sodium acetate, 1 mM EDTA | |
| **1.8% agarose gel** | 3.6 g PeqGOLD Universal Agarose, 200 µl 1X TAE, 5 µl ethidium bromide | |

The PCR products were then loaded on a 1.8% agarose gel (Table 2.9) prepared in a horizontal PerfectBlue gel system (Peqlab Biotechnologie GmbH, Erlangen) and submerged in 1X TAE buffer (Table 2.9). The DNA fragments were separated by their size in an electric field at 140 V for about 30 min and visualized under UV light by Transilluminator (Peq- Lab Biotechnologie GmbH, Erlangen).

## 2.2.3 Cell culture

All cell culture work was performed in biosafety level S2 laboratory and under safety cabinet-EN12469 (Thermo Fisher Scientific, USA). Cells were cultured in a sterile cell culture $CO_2$ incubator (Labotect, Göttingen) at 37°C, 5% $CO_2$ and 95% humidity.

The PBS used to wash the cells always refers to 1X calcium and magnesium-free PBS (Gibco®).

#### 2.2.3.1 Isolation and culture of bone marrow derived mast cells (BMMCs)

Bone marrow derived mast cells (BMMCs) were isolated from femurs and tibias of 8-9 weeks old wild-type C57BL7/6 mice. The animals were sacrificed by cervical dislocation, washed in 70% ethanol (EtOH) and then the intact bones were removed, cleaned from muscles and placed in cold Iscove's modified Dulbecco's medium (IMDM, Gibco®). Under a safety cabinet the bones were washed once with 70% EtOH and three times with PBS and then the tips of the tubular bones were cut off using fire-sterilized scissors and forceps. The bone marrow was flashed out in a 50 ml falcon tube (one tube per mouse) with IMDM using a 24 gauge needle and 20 ml syringe. The cell suspension was centrifuged at 1,300 rpm for 8 min using an Eppendorf Centrifuge 5804 (Eppendorf AG, Hamburg), the pellet re-suspended in 5 ml of IMDM and then centrifuged again. In order to lyse erythrocytes, the cell pellet was now re-suspended in 500 µl of ACK lysis buffer (150 mM $NH_4Cl$, 1 mM $KHCO_3$, 1 mM EDTA, pH 7.3 sterile filtered) and incubated for 2 min at room temperature (RT). To stop the reaction 10 ml of IMDM were added and then the suspension was filtered to remove aggregates by applying it on a 70 µm falcon cell strainer (Corning®, Kaiserslautern). After centrifugation at 1,300 rpm for 8 min the cells were re-suspended in 20 ml of IMDM supplemented with 10% fetal bovine serum (FBS) from south America origin (Gibco®), 2 mM L-glutamine (Gibco®), 1 mM pyruvate (Gibco®), 1% Penicillin/Streptomycin 10,000 U/ml (Gibco®), 100 ng/ml stem cell factor (kindly provided by Stassen M. [266]), 5 ng/ml IL-3 (PreproTech, Rocky Hill, NJ) and 50 µM 2-mercaptoethanol (Gibco®) and transferred in a cell culture flask (CELLSTAR® T-75, Greiner Bio-One, Germany). This medium promotes the differentiation of non-adherent cells into BMMCs. Floating cells were transferred in a clean flask with fresh medium twice a week until only non-adherent cells were left (about 5 weeks). BMMCs could be then used for experiments for the next 3-4 weeks.

#### 2.2.3.2 Isolation and culture of peritoneal cell-derived mast cells (PCMCs)

Peritoneal cell-derived mast cells (PCMCs) were isolated from the peritoneum of 6-9 weeks old B6, Mcpt5-Cre GRflox, alf Cre mice through peritoneal lavage. CreTG/$GR^{wt/wt}$ (GR-WT) and CreTG/$GR^{flox/flox}$ (GR-KO) animals of the same age were sacrificed by cervical dislocation and washed in 70% EtOH. Using scissors and forceps and paying attention to not to open the abdomen, the belly of the skin was removed. Ten ml of PBS at RT were injected with a 30 gauge needle into the peritoneum cavity and a short massage was done to properly wash the cavity before collecting the liquid of the lavage with a 24 gauge needle connected a 10 ml syringe. This was repeated two times. The cell suspension was collected in a 15 ml falcon tube (one per animal), centrifuged at 1,300 rpm for 10 min with an Eppendorf Centrifuge 5804 (Eppendorf AG, Hamburg), washed once with PBS and then the pellet was re-suspended in 3 ml of complete culture medium (IMDM + 10% FBS south America origin, 2 mM L-glutamine, 1 mM pyruvate, 1% Penicillin/Streptomycin, 100 ng/ml stem cell factor, 10 ng/ml IL-3 and 50 µM 2-mercaptoethanol) and plated in a 12 well plate (CELLSTAR®, Greiner Bio-One, Germany). If the pellet had a red coloration due to the presence of erythrocytes, 5 min incubation in ACK

lysis buffer and further centrifugation was performed before plating the suspension. Floating cells were transferred in a clean 12-well plate or cell culture flask, depending on the amount of cells grown, with fresh medium twice a week until only non-adherent cells were left (about 3 weeks). PCMCs could then be used for experiments for the next 2-3 weeks.

#### 2.2.3.3 Culture of RBL-2H3 cells

Rat basophilic leukemia mast cells (RBL-2H3) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco®) supplemented with 15% fetal bovine serum (FBS, Sigma-Aldrich) under standard cell-culture conditions.
Stable transfected RBL-2H3 cells were cultured in DMEM supplemented with 15% FBS and 0.6mg/ml neomycin (G418) under standard cell-culture conditions.

#### 2.2.3.4 Culture of COS-7 cells

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco®) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) under standard cell-culture conditions.

#### 2.2.3.5 Counting of cells

Adherent cells (RBL-2H3 or COS-7) were washed once with PBS, trypsinized with Trypsin-EDTA (Gibco®) for 2 min at 37°C, harvested, pelleted (800 rpm for 5 min) and re-suspended in 1 ml of medium.
Non adherent cells (BMMCs and PCMCs) were harvested, pelleted (1,300 rpm for 7 min) and re-suspended in 1 ml of medium. Ten µl (10 µl) of cell suspension was diluted 1:100 in 0.25% Trypan blue in PBS (Gibco®) and then 10 µl of this dilution was mounted in an improved Neubauer hemocytometer (Fuchs Rosenthal, 0.100 mm depth) and the number of cells was counted in 4 major quadrants using a Leica DMIL microscope (Leica Microsystems, Wetzlar, Germany). The cells number obtained by averaging the 4 quadrants was then multiplied by $10^6$ (cells/ml).

#### 2.2.3.6 Transfection of RBL-2H3 cells (electroporation)

For the transient or stable transfection of RBL-2H3 mast cells a Gene Pulser® Transfection Apparatus (BioRad, Heidelberg) for electroporation was used.
Two million ($2x10^6$) cells were suspended in 200 µl DMEM without FBS, mixed with the DNA in the amount indicated by the Table 2.10 and transferred into a 0.2 cm electroporation cuvette (BioRad, Heidelberg). The electroporation was performed at 500 µF capacitance, Extender (EXT) and 300 V. After electroporation, 800 µl of complete culture medium (see Table 2.4) were added to the cells in the cuvette, mixed gently and then the cell suspension was seeded into tissue culture dishes (CELLSTAR®, Greiner Bio-One, Germany) of appropriate size and placed in the incubator. After few hours the medium was changed to remove dead cells. The

cells could be used for experiments 48 h after electroporation in the case of transient transfection.

For stable transfection, the cells were plated in a 100 mm dish overnight after electroporation. The day after, the cells were split into five 145 mm tissue culture dishes and then two days later the medium was replaced with complete medium where 0.6 mg/ml of neomycin G418 (Sigma-Aldrich, Germany) were added to allow selection of positively transfected cells. The medium was changed with fresh one every two-three days for about two weeks during which single colonies of cells grew. After the selection period, about 20 single colonies of cells per dish were picked with a small piece of filter paper and placed into a 24-well plate to expand over a period of one month. The single colonies were checked by inverted fluorescence microscopy (Axiovert 200 - Carl Zeiss, Germany) and the positive ones confirmed by Western blot analysis (see paragraphs 2.2.4.2 - 4). Aliquots of positive transfected cells were then stored in liquid nitrogen.

#### 2.2.3.7 Transfection of COS-7 cells (PromoFectin)

Transfection of COS-7 was performed with PromoFectin (PromoCell GmbH, Heidelberg) following the manufacturer's instructions. Two µg of PromoFectin per µg of DNA (Table 2.10) were mixed in medium without FBS, incubated for 30 min at RT and then applied to the cells. The day after, the medium was changed with fresh one and the cells could be used for experiments the next day (48 h after transfection).

**Table 2.10**: Transfection type, cell type and DNA amount

| Type of transfection | Cell type | Plasmid and amount |
|---|---|---|
| Stable | RBL-2H3 | 20 µg GR-GFP MutC665A + 1 µg psV2neo |
| Transient | RBL-2H3 | 20 µg mEos2-GR |
| Transient | GR-GFP<br>GR-GFP MutC665A<br>GFP } RBL-2H3 | 4.3 µg MMTV-Luc + 0.4 µg Renilla |

| Type of transfection | Cell type | Plasmid and amount |
|---|---|---|
| Transient | COS-7 | • 1 µg GFP + 0.8µg MMTV-Luc + 0.1 µg Renilla<br>• 1 µg GR-GFP + 0.8µg MMTV-Luc + 0.1 µg Renilla<br>• 1 µg GR-GFP MutC665A + 0.8µg MMTV-Luc + 0.1 µg Renilla |

#### 2.2.3.8 Sensitization and activation of mast cells

RBL-2H3, BMMCs or PCMCs mast cells were always sensitized for 2 h with monoclonal anti-dinitrophenyl IgE (Sigma-Aldrich Chemie, Steinheim) at the final concentration of 0.5 µg/ml in complete medium. In one experiment RBL-2H3 mast cells were sensitized with Alexa647 labeled-anti-DNP IgE to be able to visualize the IgE-FcεRI clustering via fluorescence

microscopy (see paragraph 2.2.5.3). To activate the cells after 2 h of sensitization, albumin from bovine serum, 2,4-dinitrophenylated (DNP-BSA, Molecular Probes®, Thermo Fisher Scientific, USA) was added at the indicated concentration depending on the experiment and the cell type.

## 2.2.4 Protein analysis techniques

### 2.2.4.1 MAPKs phosphorylation analysis

To stimulate MAP kinases phosphorylation (Erk1/2, JNK, p38) by IgE receptor mediated activation of the cells, BMMCs or PCMCs were cultured in complete culture medium (see Table 2.4) overnight at the density of 1.5-2x$10^6$ cells/ml or 0.5x$10^6$ cells/ml respectively. The following day, the cells were treated for the indicated periods of time with Dex ($10^{-7}$ M) or vehicle EtOH (0.001%) as a negative control. Two hours before harvesting the cells, anti-DNP IgE was added in a final concentration of 0.5 µg/ml to achieve a complete loading of the FcεRI receptors with IgE molecules. About 5-10 min before the end of the 2 h, the cells were transferred to a 1.5 ml Eppendorf tube and at the time of activation they were centrifuged at 2,000 rpm for 4 min (Heraus Biofuge Pico). The medium was discarded and the cells were washed once with warmed (37°C) PBS, spun down again and re-suspended in 50 µl of warm PBS. Immediately, another 50 µl of DNP-BSA solution (0.4 µg/ml in PBS – final concentration of 200 ng/ml) were added to the cells for 15 min in a heating block (Eppendorf Thermomixer 5436) at 37°C. Note that the addition of DNP was carried out at the same time in EtOH and Dex treated samples per each time point and every 20 seconds between the different time points. The reaction was therefore stopped every 20 seconds per couple (EtOH/Dex) by lysing the cells with 100 µl of 2X SDS-sample buffer (see Table 2.11) and placing the tubes on ice.
In some experiments, the cells were treated with Dex or EtOH and, in parallel, with the GR-antagonist RU486 (1 µM, dissolved in EtOH) or the translational inhibitor cycloheximide (10 µg/ml dissolved in EtOH). These latter were added to the all samples 15 min before starting the experiment.

### 2.2.4.2 Preparation of proteins from cell lysate

The SDS-sample buffer added to the cells (see paragraph 2.2.4.1) contains β-mercaptoethanol and SDS which lead to denaturation of the proteins present in the lysate and a uniform coating with negative charge of all proteins which helps them travelling by their actual molecular weight through the SDS-PAGE gel (see next paragraph). The SDS-sample buffer lysed cells were sonicated at Amplitude 50, 4-5 pulses using a Branson Sonifier Cell disruptor B15 (G. Heinemann Ultraschall- und Labortechnik Schwäbisch Gmünd), boiled for 7-10 min at 95°C using an Eppendorf Thermomixer 5436 (Eppendorf AG, Hamburg) to allow complete protein denaturation, chilled on ice for 5 min and then centrifuged for 1 min at 10,000 rpm at 4°C using an Eppendorf Microcentrifuge 5417R (Eppendorf AG, Hamburg) before being loaded in the separation protein system.

#### 2.2.4.3 Separation of proteins by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique used to separate proteins in an electric field based on their size. The SDS-PAGE gels used in this work were made of two parts, stacking gel and separating gel that differ in the pores size: larger in the first one than the second. In the upper phase (stacking gel) the proteins migrate freely and get stacked at the interface with the second phase (separating gel). Purpose of this is to make sure that the proteins start migrate from the same level, since they have to separate only based on their mass.

For the protein analysis 8% and 10% separating polyacrylamide gels were used. 25 ml of separating gel solution (see Table 2.11) were casted between two glass plates (16 cm x 14 cm) separated by two spacers and fixed in a Cast-It M caster (Peqlab Biotechnologie GmbH, Erlangen). The solution was covered with 100% EtOH and the gel was left to polymerize at RT for 40 min. After this period the EtOH was washed away before pouring 8 ml of stacking solution (see Table 2.11) and inserting the sample comb. After polymerization (about 15 min) the comb was removed and the SDS-PAGE could be transferred into a PerfectBlue Duel Gel System Twin M (Peqlab Biotechnologie GmbH, Erlangen) and overlaid with 1X Laemmli running buffer (see Table 2.11). Generally, the first well was loaded with 5 µl of PageRuler™ Prestained Protein Ladder, 10 to 180 kDa as a marker of molecular weights (Thermo Fisher Scientific Inc., Rockford/ USA). The rest of the wells were filled with 10-30 µl of cell lysate depending on the amount of protein expected in the sample. In the empty wells was added the same amount of 1X SDS-sample buffer. An initial power of 90 V was applied for about 30 min to allow the proteins to accumulate and exceed the stacking gel, then the voltage was increased to 160 V for 3-4 h at RT. Once a sufficient separation of the proteins was achieved, the SDS-PAGE could be analyzed by immunoblotting (Western blot).

#### 2.2.4.4 Western blot analysis

After SDS-PAGE separation, the proteins were transferred from the gel to an Immobilon®-P polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Darmstadt) by applying an electric field using the wet transfer system Trans®-Blot Cell (BioRad, Heidelberg). Two filter pads, six filter papers, one methanol-activated PVDF membrane and the gel were stacked in the blotting cassette as described by manufacturer's information. The buffer tank was filled with Western blot transfer buffer (see Table 2.11), the blotting cassette was inserted and the transfer was carried out at 35 V overnight at 4°C.

The day after, the membrane was labeled, cut according with the molecular weight of the target protein and incubated for at least 30 min at RT in blocking solution (see Table 2.11) to reduce unspecific binding of antibodies. The membrane was then incubated for 3 h at RT or overnight at 4°C with specific primary antibodies (see Table 2.5). Next, three times 10 min washing of

the membrane in TBST buffer followed one another and subsequently the membrane was incubated at RT for 45 min with HRP-conjugated secondary antibodies (1:10,000 dilution in blocking solution, see Table 2.5). Blocking, washing steps and incubation with antibodies were always carried out while gently shaking. Again, three times 10 min washing of the membrane in TBST buffer was executed and then the proteins were detected by adding enhanced chemiluminescence (ECL) Western blotting substrate (BioRad, Heidelberg) as directed by the manufacturer and using a ChemiDoc Touch Imager (BioRad, Heidelberg).

#### 2.2.4.5 Membrane stripping

After detection of the proteins, the membrane could be stripped of the old bound antibodies and incubated with new primary antibodies. Normally phosphorylated proteins were detected first and then the corresponding total proteins were investigated as a proof of equal loading and reliability of the results. To perform the stripping, the membrane was incubated in stripping solution (see Table 2.11) for 10 min at 55°C while gently shaking. After this, five times 10 min washing in TBST was carried out and then the membrane was ready for blocking (10 min) and incubation with the new antibodies.

**Table 2.11:** Buffers for protein analysis

| | |
|---|---|
| **2X SDS-sample buffer** | 160 mM tris pH 6.8, 4% SDS, 20% glycerol, 12.5 mM EDTA pH 8.0, 2% ß-mercaptoethanol, 0.02% bromophenol blue |
| **10% separating gel (25ml)** | 9.9 ml $dH_2O$, 8.3 ml 30% acrylamide mix, 6.3 ml 1.5 M tris pH 8.8, 250 µl 10% SDS, 250 µl APS, 10 µl TEMED |
| **8% separating gel (25ml)** | 11.6 ml $dH_2O$, 6.7 ml 30% acrylamide mix, 6.3 ml 1.5 M tris pH 8.8, 250 µl 10% SDS, 250 µl APS, 15 µl TEMED |
| **Stacking gel (8ml)** | 5.5 ml $dH_2O$, 1.3 ml 30% acrylamide mix, 1 ml 1.5 M tris pH 6.8, 80 µl 10% SDS, 80 µl APS, 8 µl TEMED |
| **1X Laemmli running buffer** | 25 mM tris-base, 192 mM glycine, 0.1% (w/v) SDS |
| **Western blot transfer buffer** | 25 mM tris-base, 192 mM glycine, 10% MetOH |
| **1X TBST (washing buffer)** | 20 mM tris-base, 150 mM NaCl, 0,05% Tween-20 (pH 7.6) |
| **Blocking solution** | 5% (w/v) non-fat dried milk powder for total proteins or BSA powder in TBST for phosphorylated proteins |
| **Stripping buffer (50ml)** | 1 g SDS, 2.5 ml tris-HCl pH 6.8, 350 µl ß-mercaptoethanol (freshly added) |

#### 2.2.4.6 Western blot quantification

The Western blot results were optimized and quantified using Image Lab™ Software (BioRad, Heidelberg) or ImageJ.

#### 2.2.4.7 Degranulation assay: ß-hexosaminidase release measurement

As indicator of degranulation, the release of ß-hexosaminidase by IgE-DNP activated BMMCs and PCMCs was measured. To do this, 0.25-0.5x$10^6$ cells/ml/well were seeded overnight in 24-well plate (CELLSTAR®, Greiner Bio-One, Germany) and then pre-treated with Dex $10^{-7}$ M, its vehicle alone (EtOH 0.001%) or Dex in the presence of the GR-antagonist RU486 (1 µM in BMMCs, see paragraph 2.2.4.1 of the method section). The treatment was performed for indicated periods of time starting from the longest (4 h). Two hours before their activation, the cells were sensitized with monoclonal anti-dinitrophenyl IgE (anti-DNP IgE, Sigma-Aldrich, Germany) at the final concentration of 0.5 µg/ml. At the time of activation the cells, previously harvested in Eppendorf tubes, were centrifuged at 2,000 rpm for 4 min at RT using a Heraus Biofuge Pico centrifuge (Kendro, Hanau) and immediately the pellet was re-suspended in 150 µl of Tyrode's buffer with 0.1% BSA (see Table 2.12) and placed at 37°C in an Eppendorf Thermomixer 5436 (Eppendorf AG, Hamburg). Fifty µl (50 µl) of 0.1% BSA in Tyrode's buffer containing DNP-BSA (0.2 µg/ml – final concentration 50 ng/ml) were added simultaneously to EtOH and Dex of each time point every 20 sec and left for 15 min at 37°C. After 15 min, every 20 sec per couple of tubes (EtOH and Dex), the reaction was stopped by placing the tubes on ice. The cell suspension was then centrifuged at 4°C, 300 rcf for 5 min (Eppendorf Microcentrifuge 5417R) and 25 µl of supernatant pipetted in triplicates in a 96-well plate (flat bottom). Three wells with 0.1% BSA in Tyrode's buffer were added as a blank control. The rest of the supernatant was discarded, the cell pellet re-suspended in 200 µl of 0.5% Triton X-100 in Tyrode's buffer (see Table 2.12), vortexed and left for 15 min on ice to allow lysis. After centrifugation at 13,000 rpm for 5 min at 4°C (Eppendorf Microcentrifuge 5417R), 25 µl of lysate were pipetted in triplicates in another 96-well plate and three wells with 0.5% Triton X-100 were also added as a control. The lysate of PCMCs cells was conserved, 5X SDS-sample buffer was added to it to reach 1X of final concentration and be used for further Western blot analysis. Fifty µl (50 µl) of pNAG substrate (see Table 2.12) were added to the wells containing supernatant and lysate and the plates were then incubated at 37°C for about 1 h. After this time 150 µl of Glycine buffer (see Table 2.12) were added to the wells and the optical density (OD) was measured using an ultra-microplate reader ELx808IU (BioTek Instruments, Germany) at a wavelength of 415 nm. The percentage of ß-hexosaminidase released in the samples was determined by subtracting the blank controls from the corresponding sample values and then applying the formula:

$$\%\, degranulation = {}^{OD\ supernatant}\Big/_{(OD\ supernatant + OD\ lysate)} * 100$$

**Table 2.12:** Buffers for ß-hexosaminidase release

| | |
|---|---|
| **Tyrode's buffer** | 20 mM HEPES, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM Glucose (pH 7.4) + 1 mg/ml BSA (0.1%) |
| **0.5% Triton X-100 in Tyrode's buffer** | 10 ml Tyrode's buffer, 50 µl 100% Triton X-100 |
| **pNAG 1.3 mg/ml** | Dissolve 250 mg of pNAG powder in 192.3 ml of citric acid - sodium phosphate buffer (use HCl to adjust the pH to 4.5) |
| **Citric acid - sodium phosphate buffer pH 4.5** | 0.2 M $Na_2HPO_4$, 0.4 M citric acid (pH 4.5) |
| **0.2M Glycine pH 10.7** | Dissolve 1.5 g glycine in 100 ml $dH_2O$ and use NaOH to adjust the pH to 10.7 |

### 2.2.4.8 Luciferase - reporter gene assay

GR-GFP, GR-GFP MutC665A, GFP alone transfected RBL-2H3 mast cells or COS-7 cells were transfected with a Renilla luciferase expression vector (pTK Renilla Luc) and the corresponding firefly luciferase reporter (pGL3 MMTV-Luc) in the amounts described in Table 2.10. COS-7 cells were also transfected with expression vectors coding for GR-GFP, GR-GFP MutC665A and GFP constructs (see Table 2.10). The cells were seeded in 6-well plates and placed in the incubator. One day (24 h) after transfection, the cells were treated for further 24 h with the glucocorticoid Dex ($10^{-7}$ M) or its vehicle (EtOH 0.001%). After this the cells were washed twice with ice-cold PBS and lysed by adding 150 µl of 1X Passive lysis buffer (Promega, Mannheim). After 30 min on ice, the lysate was harvested in an Eppendorf tube and centrifuged at 14,000 rpm for 5 min at 4°C (Eppendorf Microcentrifuge 5417R). Twenty µl (20 µl) of each sample were then pipetted in duplicates into two 96-well plates for luciferase assay. One was used to measure the firefly luciferase activity and the other one to measure Renilla activity as an internal control of correct transfection. The luciferase activity was measured with the help of a Victor™ Light 1420 Luminescence counter (Perkin Elmer precisely, USA). To determine firefly luciferase activity, 20 µl of lysate were mixed by the machine with 70 µl of Gly-Gly reaction buffer and 20 µl of luciferin buffer (see Table 2.13). To determine Renilla luciferase activity the 20 µl of cell lysate were instead mixed with 100 µl of coelenterazine buffer (see Table 2.13) and analyzed. The luciferin and coelenterazine oxidation released luminescence were measured as relative light units (RLU) and the blank values were subtracted. Relative luciferase activity was calculated as RLU of luciferase activity divided by RLU of renilla activity.

$$Relative\ luciferase\ activity = {}^{RLU\ MMTV\ Luc}/_{RLU\ Renilla\ Luc}$$

**Table 2.13**: Buffers for luciferase assay

| | |
|---|---|
| **Gly-Gly reaction buffer** | 25 mM Glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA pH 7.8 with 1 mM DTT und 2 mM ATP |
| **Luciferin buffer** | 0.28 mg/ml 0.5 M tris, pH 7.5 |
| **Coelenterazine buffer** | 0.2 M $KH_2PO_4$, 0.2 M $K_2HPO_4$, pH 7.6 with 0.2 µM coelenterazine substrate |

## 2.2.5 Fluorescence microscopy

### 2.2.5.1 Translocation of GR-GFP into the nucleus

Hundred thousand ($1x10^5$) RBL-2H3 mast cells stably expressing GR-GFP or GR-GFP MutC665A were seeded overnight in a 2-well Nunc™Lab-Tek™Chambered Coverglass (Thermo Fisher Scientific, USA). The following day the cells were washed twice with 37°C warmed PBS and then 500 µl Tyrode's buffer with 0.1% BSA were added. The cells were then prepared under an Andor Revolution® XD spinning disk confocal laser scanning microscope (BFi OPTiLAS, Germany), which was heated at 37°C and once that the cells were focused, another 500 µl of Tyrode's buffer with 0.1% BSA containing Dex (or EtOH as a negative control) was added to reach a final concentration of $10^{-7}$ M Dex (or 0.001% of EtOH). GFP emission was recorded through a band pass filter (525±25 nm center wavelength±width, AHF, Germany) upon excitation at 488 nm. The GR translocation was acquired by taking images at every 5 sec for the first minute and then every 30 sec for up to 80 min. For the image analysis ImageJ (National Institute of Health, USA) software was used. Fluorescence intensity in the nuclei was calculated by the averaged intensity from regions identified manually. The initial intensity in the nuclei was subtracted. Subsequently, the intensities were normalized by the maximum.

### 2.2.5.2 Click-Chemistry based allergen array to activate mast cells

The micro-patterning of dots of DNP-azide onto glass slides was performed by Kumar R. using polymer pen lithography (PPL) [270]. The aim of this system setup was to visualize in real time the recruitment of GR-GFP MutC665A to the plasma membrane of activated RBL-2H3 cells and compare it to the one of GR-GFP [100].

To generate the allergen array, the glass slide was first modified (silanization+alkynization) to become "alkyne terminated" [271]. This allowed the covalent binding between the alkyne surface groups and the allergen, DNP-azide, present in the ink mixture composed by 1 µl of DNP-azide solution (0.5 mg/ml in DMSO - Jena Bioscience, Germany), 5 µl sodium ascorbate solution (20 mM in DI water - Jena Bioscience, Germany) and 5 µl $CuSO_4$ solution (10 mM in DI water - Jena Bioscience, Germany) which catalyzed the reaction called copper (I) catalyzed cycloaddition (CuAAC). Glycerol 2.75 µl were also added to the ink mixture to prevent drying. The patterning was carried out with a NLP 2000 instrument (Nanoink Inc., USA), under humidity-controlled conditions (60 to 80% relative humidity) and using a stamp where the pen array was inked with 4 µl of ink solution by spin coating (3,500 rpm, 3 min). The array was

made of 10x10 dots (1-5 µm diameter) with a distance of 10 µm between each other. After lithography, samples were kept overnight at RT to allow the click-reaction to occur and then washed with $dH_2O$ and EtOH to remove excess and unbound ink, dried with liquid nitrogen and stored dry and in the dark until used for experiment.

#### 2.2.5.3 Localization of GR at the plasma membrane

One million ($1x10^6$) GR-GFP and GR-GFP MutC665A RBL-2H3 mast cells were seeded overnight in a 60 mm cell culture dish (CELLSTAR®, Greiner Bio-One, Germany). The following day, the cells were sensitized with Alexa647 labeled anti-DNP IgE (Sigma-Aldrich, Germany) 0.5 µg/ml for 2 h. After this period of time, the cells were washed twice with PBS to remove unbound IgEs, harvested in 1ml of pre-warmed (37°C) Tyrode's buffer (see Table 2.12) with 0.1% BSA and then 100 µl of cell suspension were loaded and allowed to settle on the sample arrays for 5 or 15 min. The excess of liquid was removed and the cells attached to the pattern were fixed with 3.7% paraformaldehyde. The samples were washed once with PBS and then microscope imaging in EGFP (excitation wavelength of 488 nm - emission peak at 509 nm - green) and Cy5 (excitation wavelength of 650 nm - emission peak at 670 nm - purple) channels was carried out using an inverted fluorescence microscope TE2000 (Nikon, Germany). A 60X magnification was used to perform the imaging. First, it was focused on the FcεRI-IgE-DNP interaction visible as purple fluorescent dots corresponding to the DNP array. Second, the EGFP channel was used to detect the transfected cells (green), and of these cells, the ones showing GR co-localization with the Alexa647 IgE-DNP. The percentage of cells showing accumulation of the fluorescent GR (green dots) was calculated relative to the total number of positively transfected cells (green cells) that also showed Alexa647 IgE-DNP interaction (purple dots).

#### 2.2.5.4 Photo-activated localization microscopy (PALM)

PALM is a wide-field technique, used for single molecule tracking, based on the detection of the fluorescence emission from individual fluorescent proteins, such as mEos2, that can be photo-activated and converted by irradiation with visible light. A few fluorophores are switched on by a 405 nm laser, an image is taken, and the molecules are subsequently switched off irreversibly (photo-bleached). This procedure is repeated many times until a few thousand frames are collected. A final high-resolution image is reconstructed from the precise locations of the fluorophores in all frames, which can be determined within tens of nanometers by a two-step algorithm. First, individual fluorophores are identified within each frame (searching step), and then their centers of mass are determined (localization step) [272].
Two days (48 h) before the experiment, $2x10^6$ RBL-2H3 mast cells were transiently transfected with 20 µg of expression vector coding for mEos2-GR plasmid using electroporation (see paragraph 2.2.3.6) and seeded into 4-well Nunc™Lab-Tek™Chambered Coverglass. The day of the experiment, sensitized or non-sensitized cells as described in paragraph 2.2.3.8 were washed twice with 37°C warmed PBS and then 250 µl Tyrode's buffer (Table 2.12) with 0.1%

BSA were added. The cells were then prepared under a modified inverted total internal reflection (TIRF) microscope heated at 37°C (Axiovert 200, Zeiss, Jena, Germany). The samples were visualized with 63x1.46 oil immersion objective lens and using three diode-pumped solid state lasers combined via dichroic mirrors (AHF) and coupled into a single mode fiber (OZ Optics, Ottawa, Canada). Once that the focus with 473 nm laser (LSR473-200-T00; Laserlight, Berlin, Germany) was adjusted to visualize mEos2-GR and the PALM imaging parameters of photo-activation (162 µW, 405 nm laser - CLAS II 405-50; Blue Sky Research, Milpitas, CA) and photo-conversion (182 µW, 561 nm laser - GCL- 150-561; CrystaLaser, Reno, NV) were setup (Figure 2.2), the cells were treated by adding 250 µl of Tyrode's buffer with 0.1% BSA containing either EtOH (0.001% final concentration), Dex ($10^{-7}$ M final conc.) or Dex and DNP ($10^{-7}$ M Dex + 500 ng/ml DNP). A time lapse imaging in TIRF mode was then started taking images every 20 ms for 15 min. During the experiment, laser intensities were controlled via an acousto-optic tunable filter (AOTFnC-400.650, A-A; Opto-Electronic, Orsay, France).

PALM images of single transfected cells expressing mEos2-GR fusion proteins were acquired using an electron multiplying charge coupled device camera (Ixon DV897 BV; Andor, Belfast, UK). A total of 10,000–20,000 frames using a camera exposure time of 20 ms were recorded for each high resolution image depending on the protein expression [273]. The PALM images were analyzed by fitting a two-dimensional Gaussian distribution to the single molecule signals detected using a homemade algorithm written in Matlab (Matlab R2010b; The MathWorks, Natick, MA) [272]. For the trajectories analysis, tracking variables of 300nm of displacement (3 pixels), 3 frames of memory and at least 10 frames of goodness were considered as the same molecule. At least 3 cells per condition were analyzed.

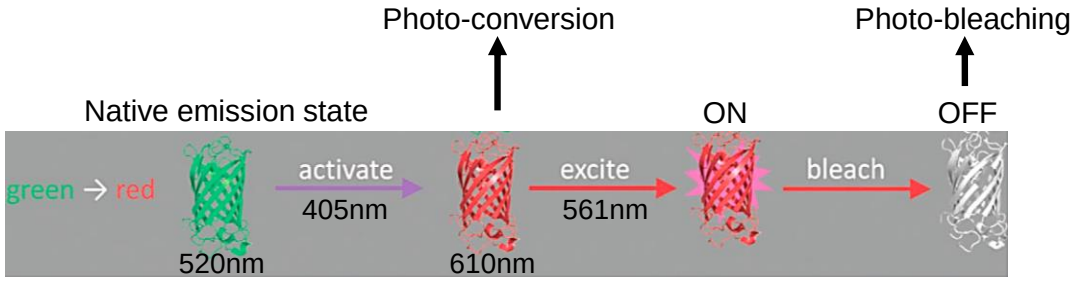


**Figure 2.2:** Scheme of PALM setup

In the native state mEos2 emits light in the green range (520 nm). Upon activation with 405 nm laser it emits light in the red range (610 nm photo-conversion) and after a light pulse of 561 nm laser it switches on and then bleaches off irreversibly (photo-bleaching). Picture modified from [274].

### 2.2.6 Statistical analysis

Unless otherwise stated, all results were expressed as mean values ± SEM. Comparisons of experimental and control groups were analyzed by Student's t-test. P-values (P) less than 0.05 were considered as statistically significant.

# 3 RESULTS

## 3.1 The action of glucocorticoids on the release of ß-hexosaminidase from BMMCs

Mast cells play a crucial role in the innate and adaptive immunity that result in inflammatory reaction associated with allergy and anaphylaxis [138, 180, 275]. Upon their activation by crosslinking of the high-affinity IgE receptors (FcεRI) on their surface or in response to pathogens, they rapidly release the content of their granules, lipid mediators and in later phase other inflammatory mediators (cytokines, chemokines) [175, 276, 277]. A large number of proteins and enzymes, mostly proteases, are contained in their granules [180, 278–280]. The glycolytic enzyme ß-hexosaminidase is also found in high amount in mast cells granules [281] and often used as a marker of degranulation [190]. GCs have been shown to rapidly attenuate allergic reactions in vivo (asthma, rheumatoid arthritis, allergic rhinitis) [93, 282] and also histamine release in RBL-2H3 and rat peritoneal mast cells within 15 min [122, 123]. To validate and characterize the rapid action of GCs in other mast cell types of different origin, the effect of the hormone on the release of ß-hexosaminidase from mouse bone marrow mast cells (BMMCs) was investigated. BMMCs were treated with vehicle (EtOH – black line) or with $10^{-7}$ M of the synthetic GC dexamethasone (Dex – red line) for the indicated periods of time prior to the time of activation of the cells. Additionally, the cells were treated with the GR antagonist (RU486 – blue line) to determine the involvement of the GR in mediating the effect of the hormone. Two hours before their stimulation, the cells were sensitized with anti-DNP IgE followed thereafter by addition of DNP-BSA for 15 min to activate them and initiate degranulation. The concentration of enzyme released was measured as a ratio of β-hexosaminidase activity in the supernatant to the whole cell lysate.

The results showed statistically significant hormone-dependent inhibition of degranulation from 1 h to 4 h and a transient, but significant GC-mediated down-regulation of degranulation at 10 min (Figure 3.1). The GR inhibitor RU486 could block both these effects of the hormone suggesting an involvement of the GR in the rapid action of GCs on inhibition of degranulation in BMMCs (Figure 3.1).

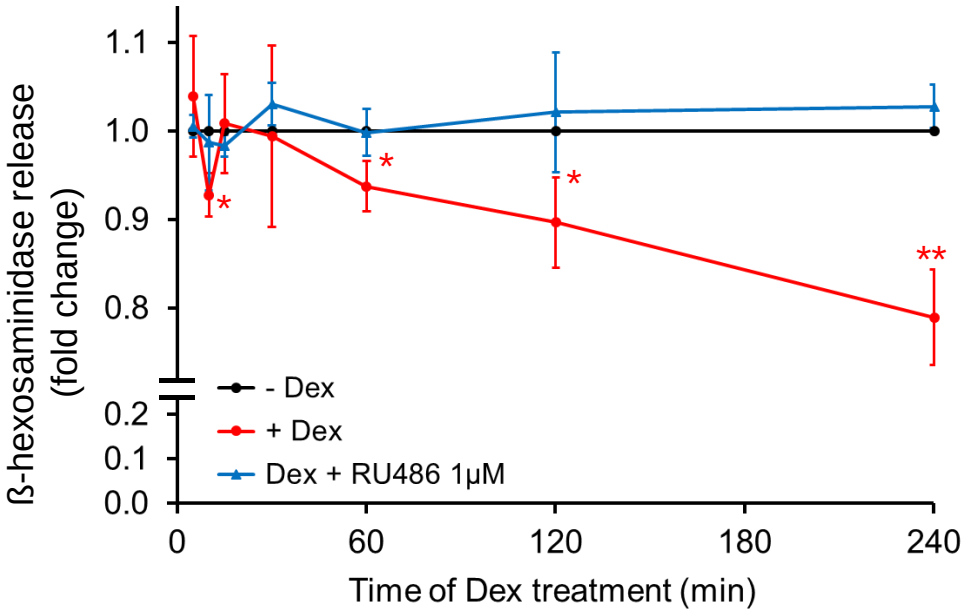


**Figure 3.1:** Effects of Dex on ß-hexosaminidase release from BMMCs

BMMCs ($0.5x10^6$ cells/ml) were seeded overnight in a 24 well plate. They were incubated for the indicated times with either the vehicle (0.001% EtOH – black line) or $10^{-7}$ M Dex (red line) in the presence (blue line) or absence of 1 µM RU486 (4 h) prior to their activation. Sensitization was carried out with 0.5 µg/ml anti-DNP IgE for 2 h before allergen (DNP-BSA 50 ng/ml) was administered for 15 min to activate them. The supernatant as well as the whole cell lysate were then analyzed for ß-hexosaminidase activity using an ELISA reader at 415 nm. *The data are expressed as means ± SEM and the statistics were generated relative to the vehicle control (n=6). * P≤0.05; ** P≤0.01.*

## 3.2 Effects of glucocorticoids on MAPKs (Erk1/2, JNK and p38) phosphorylation in BMMCs

Another downstream event of the activated mast cell pathway that is known to be inhibited in RBL-2H3 mast cells by the administration of GCs is the phosphorylation of the mitogen-activated protein kinase (MAPK) Erk1 and Erk2 [100, 257, 261].

To determine whether phosphorylation of the MAPKs Erk1 and Erk2, but also JNK and p38 is rapidly affected by the action of Dex in the activated primary mast cells, lysates from BMMCs were subjected to Western blot analysis. The cells were treated with vehicle (EtOH) or with $10^{-7}$ M Dex for different periods of time starting from 4 h prior to the activation of the cells. Two hours before their activation, the cells were sensitized with anti-DNP IgE and thereafter activated with DNP-BSA for 15 min which stimulated phosphorylation of the MAPKs. To detect the phosphorylation state of the proteins, phospho-specific Erk1/2, JNK or p38 antibodies were used, while non-phosphorylation specific Erk1/2 or p38 antibodies were utilized as the loading controls.

The results of the Western blot analysis indicated a transient GC-dependent up-regulation of the phosphorylation of Erk1/2 between 5 and 10 min, while JNK and p38 phosphorylation were not affected by hormone administration (Figure 3.2 A). However, this transient rapid effect of GC on Erk1/2 in BMMCs varied immensely from experiment to experiment in terms of the

intensity of the effect and the time at which it occurred. Since Erk1/2 phosphorylation has recently been used as a parameter to investigate the rapid action of GCs in mast cells [100], the effect of Dex on Erk1/2 phosphorylation was quantified from five independent experiments. As the bar chart shows, no hormone effects on Erk1/2 phosphorylation from 5 to 15 min were found to be statistically significant (Figure 3.2 B). The transient GC-dependent up-regulation of Erk1/2 phosphorylation was therefore considered a pleiotropic action of the hormone and was not further analyzed. On the other hand, a constant Dex-mediated down-regulation of the phosphorylation of Erk1/2, JNK and, to a lesser extent, of p38 was observed by Western blot analysis starting from 30 min to 4 h of hormone administration (Figure 3.2 C). Moreover, the inhibition of Erk1/2 phosphorylation induced by Dex from 30 min was statistically significant as shown in Figure 3.2 D.

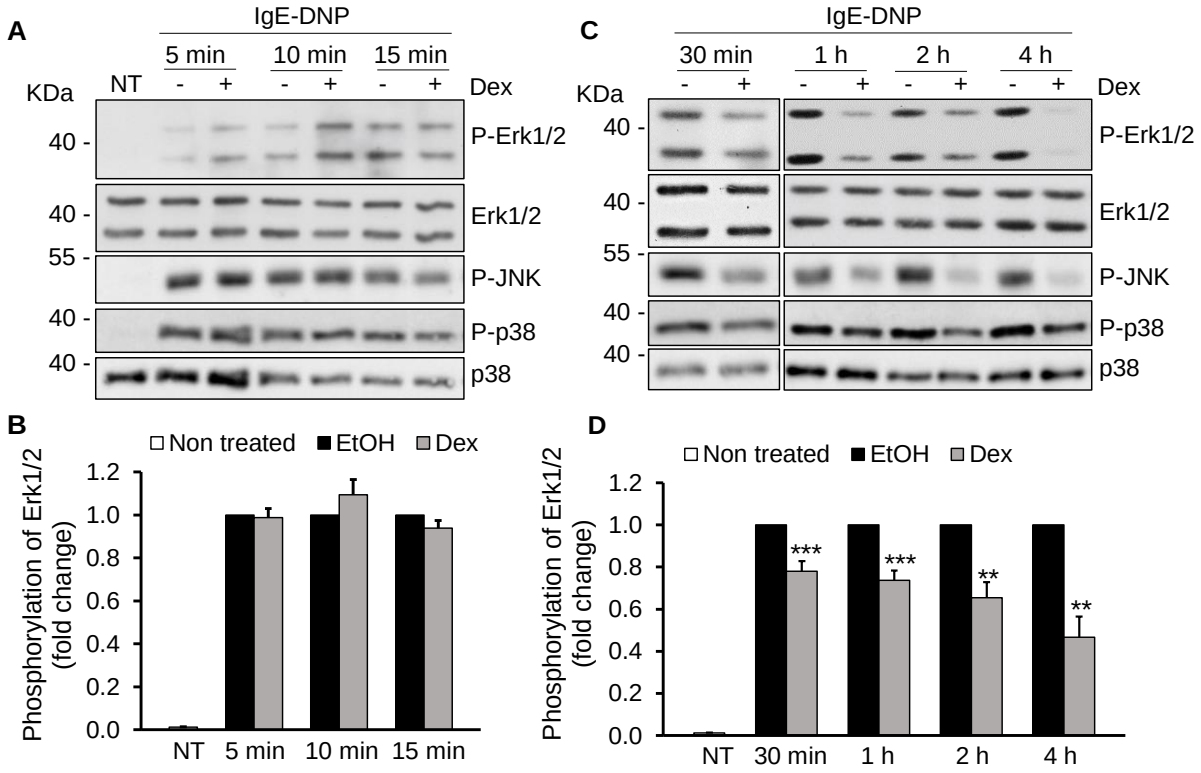


**Figure 3.2:** Effects of Dex on phosphorylation of MAPKs in BMMCs

**(A and C)** Western blot analysis: BMMCs (1.5-2x10$^6$ cells/ml) were seeded overnight in a 24 well plate. The cells were incubated for the indicated times with vehicle (0.001% EtOH) or with $10^{-7}$ M Dex prior to their activation. They were sensitized with 0.5 µg/ml anti-DNP IgE for 2 h before allergen (DNP-BSA 200 ng/ml) was applied for 15 min to activate them. The immunoblots were incubated with specific anti-phospho MAPKs (Erk1/2, JNK or p38) antibodies. Protein loading was determined by incubation of the membranes with phosphorylation state independent Erk1/2 or p38 antibodies. **(B and D)** Quantification of phosphorylation of Erk1/2 in the absence (black bars) or presence (gray bars) of Dex. *The data are normalized relative to the loading control and expressed as means ± SEM (n=5). ** P≤0.01; *** P≤0.001.*

### 3.2.1 Glucocorticoid-mediated regulation of Erk1/2 activation in BMMCs requires the GR and de novo protein synthesis

To determine whether the rapid inhibition of Erk1/2 phosphorylation observed from 30 min is dependent on the GR, the GR antagonist RU486 was used to perform experiments in which the BMMCs were treated either with vehicle (EtOH) as a negative control or with 1 µM RU486 for 4 h. During this time the cells were also incubated with vehicle (EtOH) or with $10^{-7}$ M Dex for different periods of time starting from 4 h. Two hours before their activation, the cells were sensitized with anti-DNP IgE and subsequently activated with DNP-BSA for 15 min to stimulate Erk1/2 phosphorylation. To detect the phosphorylation state of the protein, phospho-specific Erk1/2 antibodies were used. Non-phosphorylation specific Erk1/2 antibodies were utilized as the loading control. Moreover, Erk1/2 phosphorylation events from three independent experiments were quantified. The results were normalized relative to the signal of the loading control. A significant GC-mediated down-regulation of the activation of Erk1/2 from 30 min to 4 h in the absence of RU486 was observed and was completely blocked by the addition of the GR antagonist (Figure 3.3 A and B). The GR is therefore required to mediate this rapid action of GCs in BMMCs.

To determine whether the rapid inhibitory effect of GCs on Erk1/2 phosphorylation is a primary or secondary action of the hormone, the protein synthesis inhibitor cycloheximide (CHX) or the vehicle (EtOH), were added to the BMMCs for 4 h. The cells were also treated with vehicle (EtOH) or with $10^{-7}$ M Dex for different periods of time prior to their sensitization with anti-DNP IgE for 2 h and their activation with DNP-BSA for 15 min to stimulate the activation of the MAPK. Western blot analysis of the lysates was carried out and Erk1/2 phosphorylation signals from three independent experiments were quantified. The results were normalized relative to the signal of the loading control. In the absence of CHX, the inhibitory effect of Dex on phosphorylation of Erk1/2 was found significant starting from 30 min to 4 h of pre-treatment of the cells. This effect was abolished by the addition of the translational inhibitor (CHX) (Figure 3.4 A and B).

These results together suggest that the GC down-regulation of Erk1/2 activation from 30 min is not only dependent on the GR, but it also requires de novo protein synthesis, indicating that it is most likely driven by genomic mechanism of the hormone.

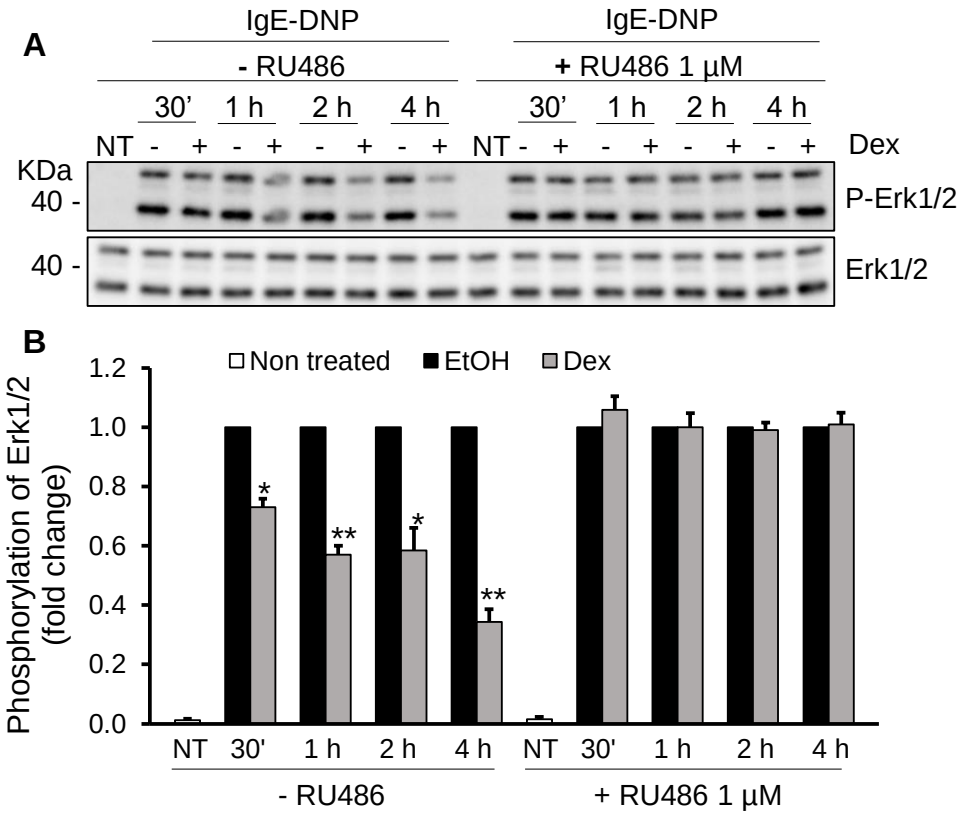


**Figure 3.3:** Effects of RU486 on GC-mediated regulation of Erk1/2 activation in BMMCs

**(A)** Western blot analysis: BMMCs (1.5-2x$10^6$ cells/ml) were seeded overnight in a 24 well plate. The cells were incubated for the indicated times with vehicle (0.001% EtOH) or with $10^{-7}$ M Dex in the presence or absence of 1 µM RU486 (4 h) prior to their activation. They were sensitized with 0.5 µg/ml anti-DNP IgE for 2 h before allergen (DNP-BSA 200 ng/ml) was applied for 15 min to activate them. The immunoblots were incubated with specific anti-phospho Erk1/2 antibodies. Protein loading was determined by incubation of the membranes with non-phosphorylation specific Erk1/2 antibodies. **(B)** Quantification of the phosphorylation of Erk1/2 in the absence (black bars) or presence (gray bars) of Dex and in the absence (left) or presence (right) of RU486 from three independent experiments. *The data are normalized relative to the loading control and expressed as means ± SEM. * P≤0.05; ** P≤0.01.*

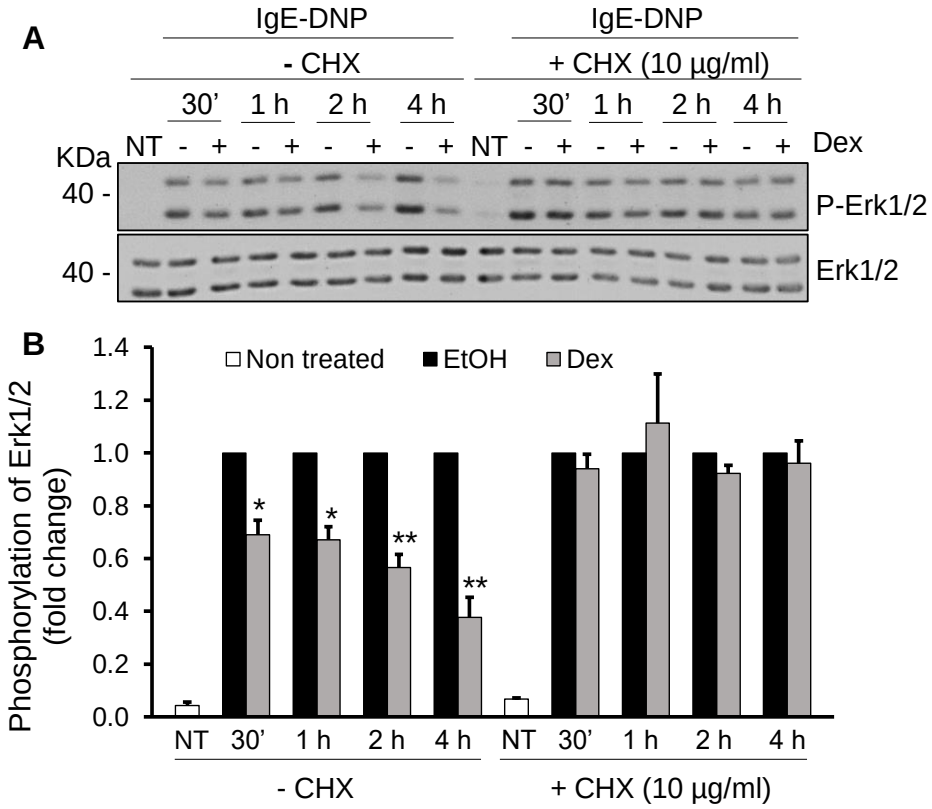


**Figure 3.4:** Effects of CHX on GC-mediated regulation of Erk1/2 activation in BMMCs

**(A)** Western blot analysis: BMMCs (1.5-2x$10^6$ cells/ml) were seeded overnight in a 24 well plate. The cells were incubated for the indicated times with vehicle (0.001% EtOH) or with $10^{-7}$ M Dex in the presence or absence of 10 µg/ml CHX (4 h) prior to their activation. They were sensitized with 0.5 µg/ml anti-DNP IgE for 2 h before allergen (DNP-BSA 200 ng/ml) was applied for 15 min to activate them. The immunoblots were incubated with specific anti-phospho Erk1/2 antibodies. Protein loading was determined by incubation of the membranes with non-phosphorylation specific Erk1/2 antibodies. **(B)** Quantification of the phosphorylation of Erk1/2 in the absence (black bars) or presence (gray bars) of Dex and in the absence (left) or presence (right) of CHX from three independent experiments. *The data are normalized relative to the loading control and expressed as means ± SEM. * P≤0.05; ** P≤0.01.*

## 3.3 Effects of glucocorticoids on Erk1/2 phosphorylation in PCMCs

To conclusively demonstrate the involvement of the GR in the rapid action of GCs in mast cells, a specific tissue knock-out of the receptor was generated through Cre/loxP recombination system in peritoneal cell-derived mast cells (PCMCs). In this system Cre-recombinase enzyme is expressed under the control of the promoter Mcpt-5 which is active in mature connective tissue mast cells like PCMCs, but not in mucosal tissue mast cells or mast cells progenitors like BMMCs. PCMCs but not BMMCs were therefore selectively knocked out for GR and this occurred when the cells expressed Cre-recombinase enzyme at the same time as GR flanked by two loxP sites. These sites are recognized by the Cre enzyme which can cut out the gene making it inactive [161, 267, 283].

To be sure that GCs behave the same way in PCMCs as in BMMCs, the GC regulation of Erk1/2 activation was investigated in wild-type PCMCs. The effects of Dex on Erk1/2 phosphorylation were also validated with the protein synthesis inhibitor CHX in cells obtained from GR-WT mice. Wild-type PCMCs were treated either with vehicle (EtOH) as a negative control or with 10 µg/ml CHX for 4 h. During this time the cells were also incubated with vehicle (EtOH) or with $10^{-7}$ M Dex for different periods of time starting from 4 h. Two hours before their activation, the cells were sensitized with anti-DNP IgE and finally activated with DNP-BSA for 15 min. The cells were then lysed and Western blot analysis was performed using phospho-specific Erk1/2 antibodies. Non-phosphorylation specific Erk1/2 antibodies were utilized as the loading control.
In the absence of CHX, treatment of PCMCs with Dex was able to inhibit Erk1/2 phosphorylation starting from 1 h. At 5, 10 and 15 min no effects of Dex were observed (Figure 3.5 left panel). Moreover, the effect of the hormone from 1 h was abolished by the addition of the translational inhibitor CHX (Figure 3.5 right panel), indicating the necessity of de novo protein synthesis for the action of Dex in these cells.

Thus, the effects of Dex on Erk1/2 phosphorylation in PCMCs were delayed (1 h) compared to BMMCs (30 min). In accordance with BMMCs, the effects of Dex in PCMCs were not mediated by a primary action of the hormone on the kinase, but they required the protein synthesis and therefore they were most likely driven by genomic mechanism of action of GC.

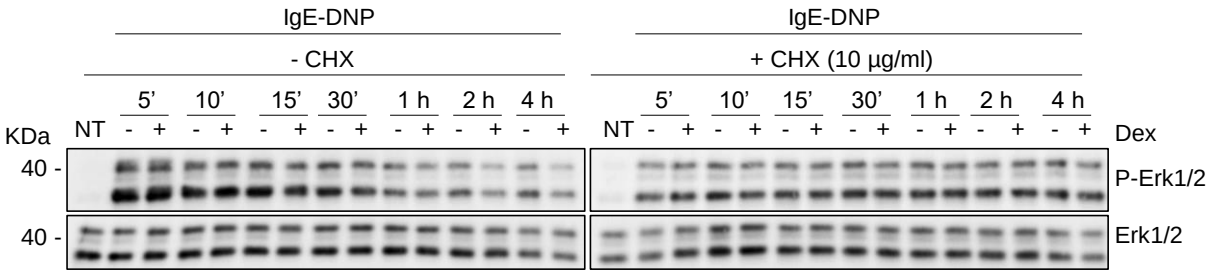


**Figure 3.5:** Effects of CHX on GC-mediated regulation of Erk1/2 activation in PCMCs

Western blot analysis: PCMCs ($0.25x10^6$ cells/ml) from GR-WT mice were seeded overnight in a 24 well plate. The cells were incubated for the indicated times, prior to their activation, with vehicle (0.001% EtOH) or with $10^{-7}$ M Dex in the presence or absence of 10 µg/ml CHX (4 h). They were sensitized with 0.5 µg/ml anti-DNP IgE for 2 h before allergen (DNP-BSA 50 ng/ml) was administrated for 15 min to activate them. The cells were lysed and immunoblotted using anti-phospho Erk1/2 antibodies. Protein loading was determined by incubating the membranes with anti-Erk1/2 antibodies.

### 3.3.1 Involvement of the GR in the Erk1/2 phosphorylation in PCMCs

To confirm the involvement of the GR in the effects mediated by Dex on Erk1/2 phosphorylation, Western blot analysis of the phosphorylation status of Erk1/2 following hormone treatment was carried out in the GR knock-out PCMCs.

As a first step, the actual lack of GR expression was verified by Western blot analysis which was carried out running the lysate of $2x10^6$ PCMCs from GR-KO mice compared to the same amount of cells from GR-WT mice. Specific anti-GR antibodies were utilized to detect the receptor protein, while anti-ß-actin antibodies were used as the loading control. The Western blot results showed a knock-out of the GR, since the GR signal which was seen in the PCMCs isolated from the wild-type mice was not detectable in the cells isolated from the GR-KO PCMCs (Figure 3.6 A). PCMCs from confirmed GR-WT or GR-KO mice were treated with vehicle (EtOH) or with $10^{-7}$ M Dex for the indicated periods of time starting from 4 h prior to the activation of the cells. Two hours before activation, the cells were sensitized with anti-DNP IgE and thereafter treated with DNP-BSA for 15 min to stimulate MAPKs phosphorylation. Western blot analysis was carried out on phosphorylation of Erk1/2 for both GR-WT and GR-KO cells and the results were quantified relative to the signals of non-phospho specific Erk1/2 antibodies.

As observed for the BMMCs, the effect of Dex on Erk1/2 phosphorylation in PCMCs at 10 min was not statistically significant (Figure 3.6 B left panel and C red bars). Remarkably, the inhibitory effect of Dex seen in BMMCs from 30 min onwards was also detected in PCMCs, but with a lag time of another 30 min making the inhibition clearly visible and statistically significant only after 1 h (Figure 3.6 B left panel and C red bars). The cells from the GR-KO mice, however, showed no Dex effect on Erk1/2 phosphorylation (Figure 3.6 B right panel and C blue bars), suggesting that the receptor is required for the action of the hormone.

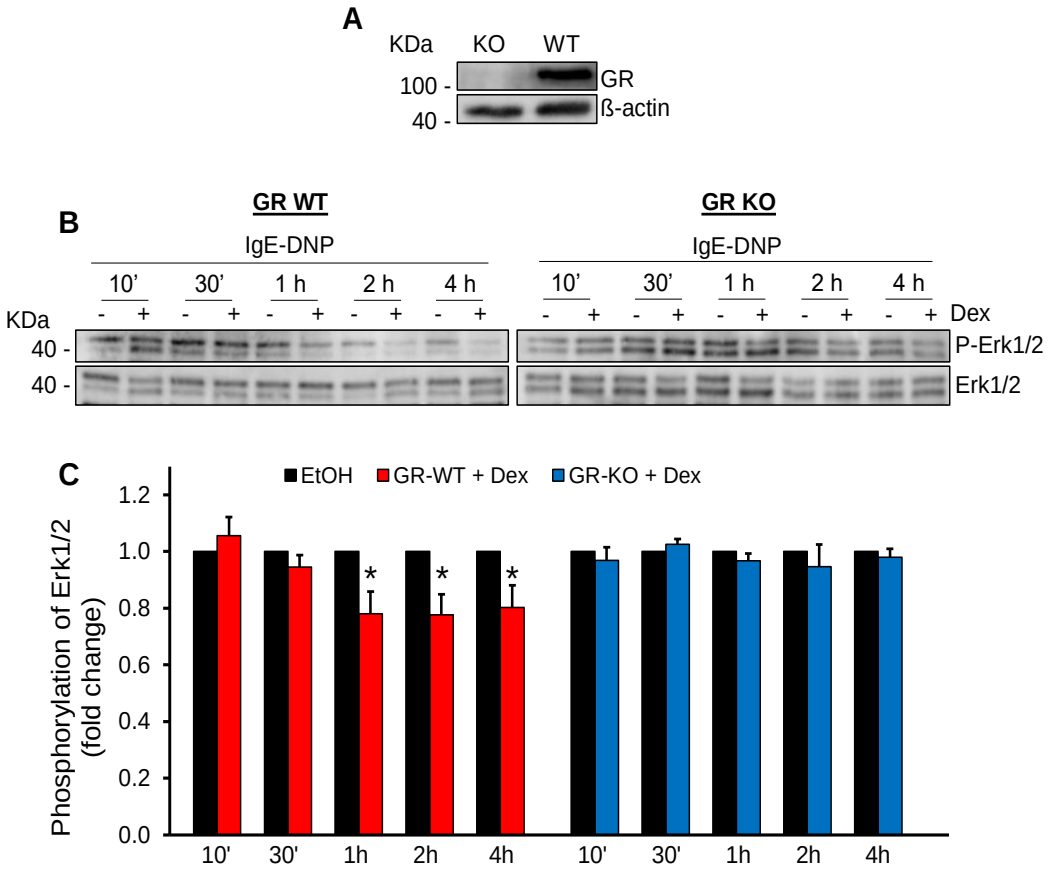


**Figure 3.6:** Effects of Dex on phosphorylation of Erk1/2 in PCMCs

**(A)** Control Western blot showing that the GR is knocked out of the PCMCs of GR-KO mice (left) compared to cells from GR-WT mice (right). PCMCs ($2x10^6$) were lysed and immunoblotted using specific anti-GR antibodies. Anti-ß-actin antibodies were used as the loading control.
**(B)** Western blot analysis of phosphorylation of MAPKs: PCMCs ($0.25x10^6$ cells/ml) from GR-WT or GR-KO mice were seeded overnight in a 24 well plate. The cells were incubated for the indicated times with vehicle (0.001% EtOH) or with $10^{-7}$ M Dex prior to the activation. They were sensitized with 0.5 µg/ml anti-DNP IgE for 2 h before allergen (DNP-BSA 50 ng/ml) was administrated for 15 min to activate them. The cells were lysed and run on a SDS-PAGE gel followed by immunoblotting with specific anti-phospho Erk1/2 antibodies. Protein loading was determined by incubation of the membranes with phosphorylation state independent Erk1/2 antibodies. **(C)** Quantification of the Erk1/2 phosphorylation signals of the PCMCs from GR-WT (red bars) and from GR-KO (blue bars) mice in the absence (black bars) or presence (red/blue bars) of Dex. *The data are normalized relative to the loading control and expressed as means ± SEM (n=4). * P≤0.05.*

## 3.4 Effects of glucocorticoids on the release of ß-hexosaminidase from PCMCs

To further confirm that the GR mediates the rapid effects of GCs in PCMCs, the effect of the hormone on the degranulation of activated mast cells, isolated from GR-WT and GR-KO mice was analyzed.

As previously described for BMMCs, the PCMCs from GR-WT and GR-KO mice were treated with vehicle (EtOH – black line) or with $10^{-7}$ M Dex (GR-WT indicated by red line, GR-KO by blue line) for different periods of time starting from 4 h prior to the activation of the cells. Two hours before the activation, the cells were sensitized with anti-DNP IgE and thereafter were activated with DNP-BSA for 15 min to stimulate the release of the granules content. Degranulation was measured as the ratio of β-hexosaminidase activity in the supernatant over the cell lysate. Similar to the situation in BMMCs, the results showed a statistically significant hormone-dependent inhibition of degranulation from 1 h to 4 h and a transient, but still significant GC-dependent down-regulation of degranulation at 15 min (Figure 3.7 – red line). These effects were completely absent in the GR-KO cells where the hormone did not show any activity on the modulation of ß-hexosaminidase release (Figure 3.7 B – blu line).

Thus, Dex showed in PCMCs the same effects seen in BMMCs both on Erk1/2 phosphorylation and the release of ß-hexosaminidase. However, these effects were delayed in PCMCs compared to BMMCs. Moreover, the GR knock-out in PCMCs confirmed the receptor as a mediator of the hormone effects.

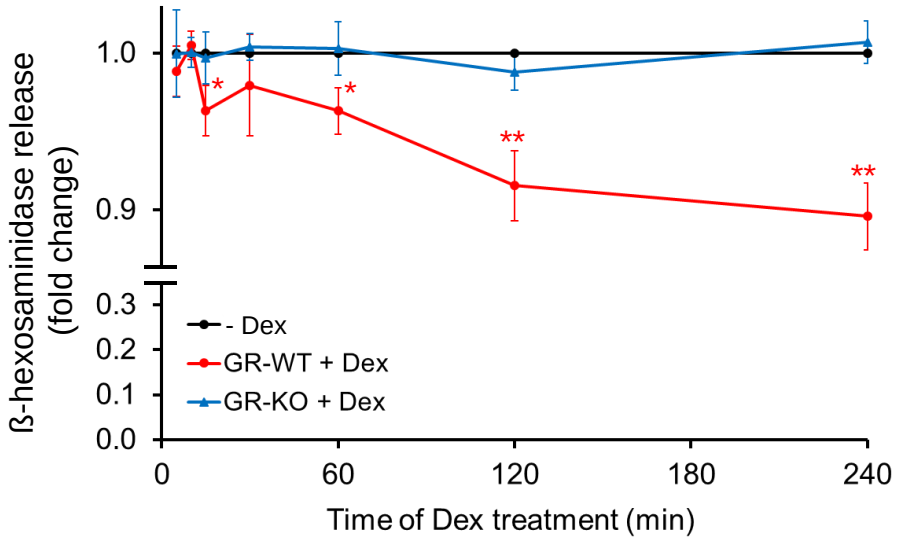


**Figure 3.7:** Effects of Dex on ß-hexosaminidase release from PCMCs

ß-hexosaminidase release expressed in fold change relative to the EtOH control: PCMCs ($0.25x10^6$ cells/ml) from GR-WT or GR-KO mice were seeded overnight in 24 well plate. The cells were incubated for the indicated times with vehicle (0.001% EtOH – black line) or with $10^{-7}$ M Dex (red or blue line) prior to the activation. They were sensitized with 0.5 µg/ml anti-DNP IgE for 2 h and thereafter activated with allergen (DNP-BSA 50 ng/ml) for 15 min. The supernatant as well as the whole cell lysate were then analyzed for ß-hexosaminidase content using an ELISA reader at 415nm. *The data are expressed as means ± SEM and the statistics were generated relative to the vehicle control (n=4). * P≤0.05; ** P≤0.01.*

## 3.5 Membrane localization of the GR as a site for the rapid action of glucocorticoids

The results from BMMCs and PCMCs demonstrated a rapid GC-mediated down-regulation of degranulation at 10 - 15 min and a rapid GC inhibition of Erk1/2 phosphorylation at 30 - 60 min. As both processes are dependent on the GR, the question arises as to how the receptor could exert its rapid effects at 10 - 15 min since the later at 30 - 60 min were demonstrated to likely be genomic effects of the hormone. A possibility is that the 10 - 15 min effects are mediated by a membrane localization of the GR as it has previously been reported [100]. One example of how a protein like the GR can interact with the plasma membrane, is through the covalent attachment of a cysteine in the receptor to membrane fatty acids via palmitoylation. As Cys665 in the GR has been suggested to be a putative palmitoylation site because of its homology to a motif in hERα (see paragraph 1.2.5.1 of the introduction), the likely involvement of this site in the membrane localization of the GR was investigated. To do this, RBL-2H3 mast cells were chosen instead of BMMCs or PCMCs because they are relatively easy to transfect compared to the primary mast cells. Position 665 of the human GR was mutated from a cysteine to an alanine and the mutant was stably transfected into the cells as a fusion with green fluorescent protein (GR-GFP MutC665A). Its action was then compared with the one of a wild-type receptor (GR-GFP) also stably transfected in the same cell line. To determine whether the classical GR response was impaired by the mutation, nuclear localization and transactivation functions of the receptor were analyzed.

GR-GFP and GR-GFP MutC665A stably transfected RBL-2H3 mast cells were seeded overnight and then subjected to real-time microscopy studies in the presence of $10^{-7}$ M Dex to analyze the nuclear localization of the receptor. Treatment of the cells with vehicle (EtOH) was used as a negative control. A time lapse imaging of the translocation of the receptor into the nucleus was measured from 0 to 80 min following vehicle or hormone administration. The results showed no nuclear translocation of both GR-GFP and GR-GFP MutC665A upon vehicle treatment (Figure 3.8 A panels 1 and 3). Upon $10^{-7}$ M Dex treatment, nuclear translocation of GR-GFP was complete after about 20 min, while that of the GR-GFP MutC665A was delayed and was still incomplete after 80 min (Figure 3.8 A panels 2 and 4). The quantification of the nuclear translocation of the fluorescent GR or mutant GR is represented in Figure 3.8 B where the percentage of fluorescence intensity in the nucleus of GR-GFP or GR-GFP MutC665A transfected cells was plotted over the time of hormone treatment. No vehicle control is shown in Figure 3.8 B since no nuclear translocation was observed following this treatment (negative control).

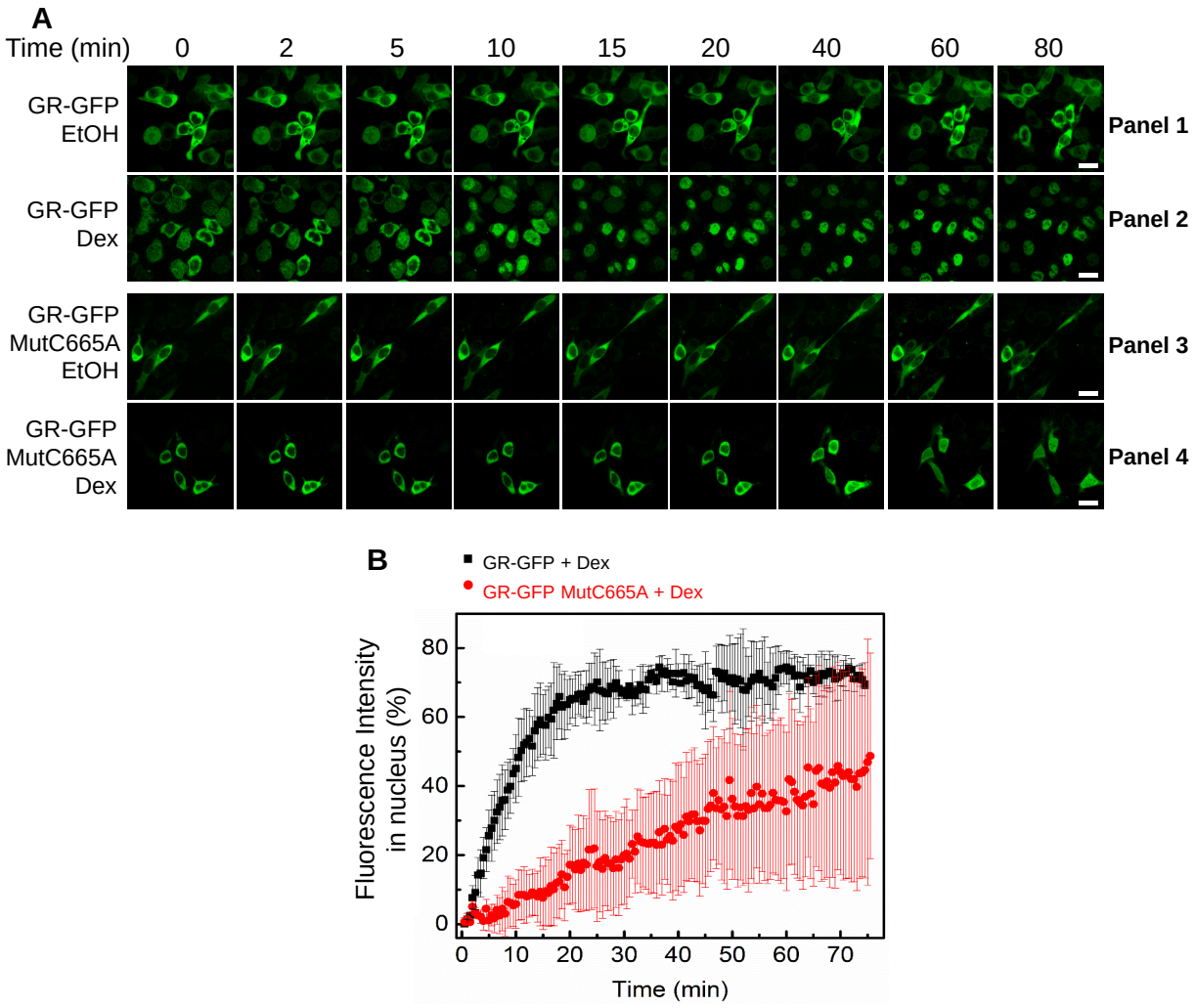


**Figure 3.8:** Effect of C665A mutation on GR nuclear translocation in RBL-2H3 cells

Nuclear translocation of GR was investigated in RBL-2H3 mast cells stably transfected with wild-type GR fused to GFP (GR-GFP) or mutant "C665A" GR fused to GFP (GR-GFP MutC665A) using spinning disk confocal laser scanning microscopy. In **(A)**: 80 min time lapse imaging showing nuclear translocation of WT (panels 1 and 2) or MutC665A (panels 3 and 4) GR upon vehicle (0.001% EtOH – panels 1 and 3) or $10^{-7}$M Dex (panels 2 and 4) treatment. Scale bar is the same in all the figures and corresponds to 10 µm. **(B)** Quantification of the nuclear translocation of the fluorescent GR or mutant GR from the experiment in A: Kinetics of the nuclear translocation of GR-GFP (black spots) or GR-GFP MutC665A (red spots) in transfected RBL-2H3 cells upon treatment with $10^{-7}$ M Dex.

Reporter gene assay analysis was also carried out to investigate the ability of the mutant GR to activate target genes in comparison to the wild-type receptor. First, Western blot analysis of the GR expression in the stably transfected RBL-2H3 mast cells was performed. Specific anti-rat GR and anti-human GR antibodies were employed to detect the endogenous receptor and the transfected one (human GR fused to GFP) respectively, while anti-ß-actin antibodies were used as the loading control. As Figure 3.9 A shows, GFP alone transfected cells expressed only the endogenous GR, while the GR-GFP and mutant “C665A” GR-GFP (GR-GFP MutC665A) transfected cells expressed both the endogenous and the transfected GR. The mutant GR-GFP was expressed more than the wild-type GR-GFP. RBL-2H3 mast cells stably expressing GR-GFP, GR-GFP MutC665A and GFP alone were transiently transfected with murine mammary tumor virus-luciferase (MMTV-luc) reporter gene that carries the glucocorticoid response element (GRE), driving the expression of a firefly luciferase gene. Renilla luciferase reporter gene construct was co-transfected as an internal control for the transfection efficiency. Twenty-four hours after transfection, the cells were treated with vehicle (EtOH) or with $10^{-7}$ M Dex for an additional 24 h and then lysed and assayed for luciferase activity.

Upon Dex administration, GFP alone expressing RBL-2H3 mast cells significantly transactivated with an induction factor of 15 ± 6 relative to the vehicle control (Figure 3.9 B). This was possibly due to the background activity of the endogenous GR. The cells expressing GR-GFP induced a stronger luciferase activity than the cells expressing GFP, with an induction factor of 61 ± 19 (Figure 3.9 B). This was expected but most likely due to the synergistic action of endogenous and transfected receptors. The mutation C665A of the GR did not prevent the transactivation ability of the receptor. Moreover, the transactivation by the mutant GR resulted not significantly different from the transactivation by the wild-type GR (Figure 3.9 B). This was possibly due to the high expression level of GR-GFP MutC665A compared to GR-GFP, as shown by the Western blot analysis in Figure 3.9 A. Additionally, the GR-GP MutC665A induction factor of 236 ± 133 was characterized by a very large error range that most likely affected the statistical analysis. This high variability from experiment to experiment was probably a result of the transactivation function of the endogenous receptors expressed by the cells.

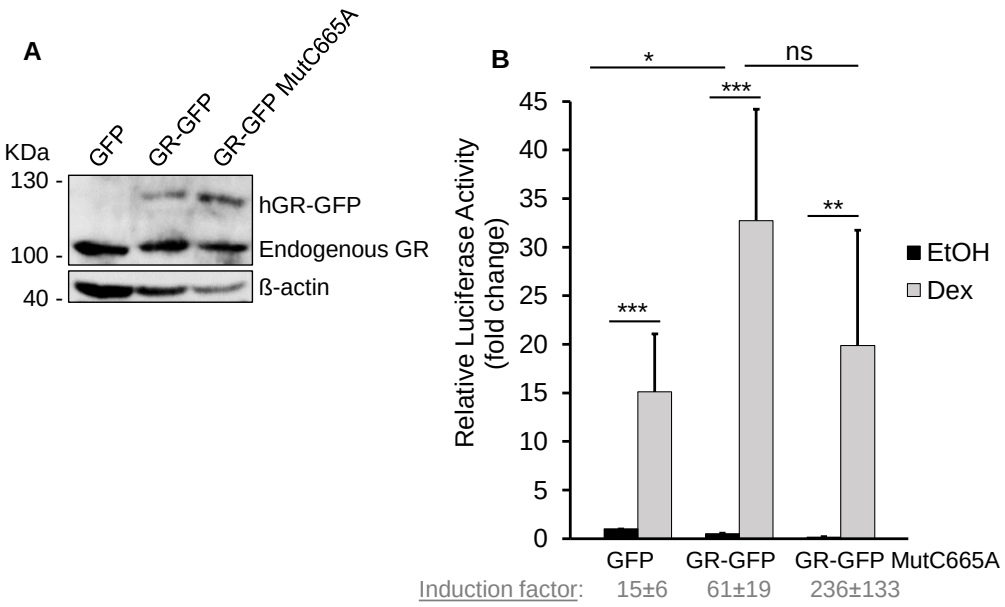


**Figure 3.9:** Effect of GR C665A mutation on transactivation by the GR in RBL-2H3 mast cells

**(A)** RBL-2H3 mast cells ($2x10^6$) which stably expressed GFP alone, GR-GFP or GR-GFP MutC665A were lysed and immunoblotted. Specific anti-rat GR and anti-human GR antibodies were used to detect the endogenous or the transfected GR (h GR-GFP) respectively. Anti-ß-actin antibodies wer used as the loading control. **(B)** Luciferase reporter gene assay to assess the impact of the mutation C665A on the GR transactivation. RBL-2H3 mast cells clones shown in A were co-transfected with murine mammary tumor virus-luciferase (MMTV-luc) reporter gene construct and a plasmid expressing Renilla luciferase enzyme as a control of transfection efficiency. The cells were treated with vehicle (0.001% EtOH – black bars) or $10^{-7}$ M Dex (gray bars) for 24 h and luciferase activity was measured. Shown are the bar charts of the results expressed in fold change relative to the vehicle control of GFP. *Data are means ± SEM (n=3). * P≤0.05; ** P≤0.01; *** P≤0.001; ns=not significant.*

### 3.5.1 Effect of C665A mutation on nuclear translocation and transactivation of the GR in COS-7 cells

The RBL-2H3 mast cells used in the previous nuclear translocation and transactivation experiments express endogenous GR which might have had an impact on the properties of the transfected receptor and therefore affecting the results of the experiments. To exclude any endogenous GR activity, the same experiments were repeated in COS-7 cells that do not express endogenous GR.

In the first analysis, COS-7 cells were transiently transfected with expression vectors coding for GR-GFP or GR-GFP MutC665A for 48 h. After this time the cells were treated with the vehicle (EtOH) or $10^{-7}$ M Dex and immediately subjected to time lapse imaging for 60 min using confocal microscopy.

The wild-type receptor showed rapid nuclear translocation upon hormone treatment that was complete after between 20 to 40 min. As expected, the mutant GR demonstrated a delayed time of translocation into the nucleus upon Dex treatment that was still incomplete at 60 min (Figure 3.10).

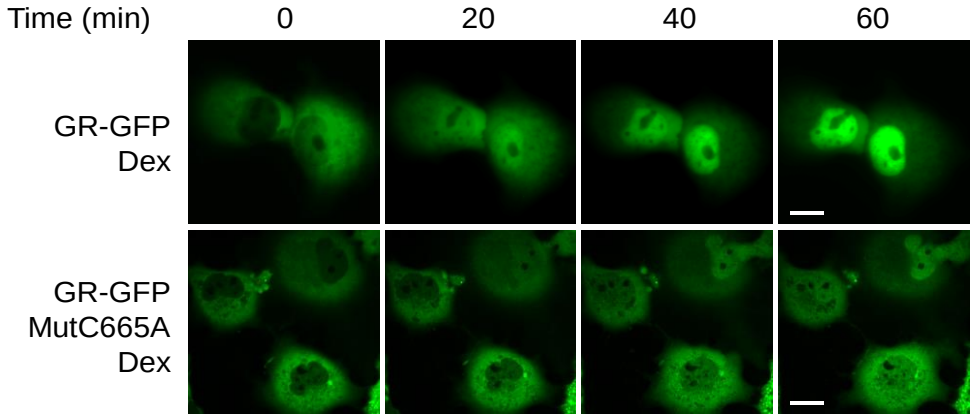


**Figure 3.10:** Effect of C665A mutation on GR nuclear translocation in COS-7 cells

Nuclear translocation of GR was investigated using spinning disk confocal laser scanning microscopy in COS-7 cells transfected with GR-GFP or GR-GFP MutC665A constructs. The figure shows 60 min time lapse images of the nuclear translocation of wild-type (upper panel) or MutC665A (lower panel) GR upon $10^{-7}$M Dex treatment. Scale bar is the same in all the figures and corresponds to 10 µm.

In the second analysis, COS-7 cells were transiently transfected with expression vectors coding for GR-GFP, GR-GFP MutC665A or GFP alone and also co-transfected with murine mammary tumor virus-luciferase (MMTV-luc) and Renilla luciferase reporter genes. Western blot analysis was carried out to show the expression of GR. Specific anti-human GR antibodies were utilized to detect the transfected receptor, while anti-ß-actin antibodies were used to check for equal protein loading. Figure 3.11 A shows that GFP alone transfected COS-7 cells did not express GR, while both GR-GFP and GR-GFP MutC665A transfected cells expressed the receptor. However, the expression level of the receptor was higher in the mutant than the

wild-type GR transfected cells. Twenty-four hours after transfection, COS-7 cells were treated with vehicle (EtOH) or with $10^{-7}$ M Dex for an additional 24 h and then lysed and assayed for luciferase activity.

The results reported in Figure 3.11 B show that in GFP alone transfected cells, where the GR was not present, the hormone hardly showed an increase in the activity of the reporter plasmid (induction factor of 1.4 ± 0.3 relative to the vehicle control). On the contrary, upon Dex treatment, the GR-GFP expressing cells showed significantly increased transactivation compared to the GFP alone transfected cells, with an induction factor of 21.5 ± 4.7. On the other hand, although the transfected COS-7 cells expressed an higher level of mutant GR than wild-type GR (Figure 3.11 A), as also seen in RBL-2H3 cells, GR-GFP MutC665A significantly enhanced transactivation upon hormone treatment, with an induction factor of 4.7 ± 1.2, but the overall transactivation ability was significantly reduced compared to the wild-type receptor (Figure 3.11 B).

Thus, the mutation C665A of the GR delays its nuclear translocation and reduces its gene transactivation.

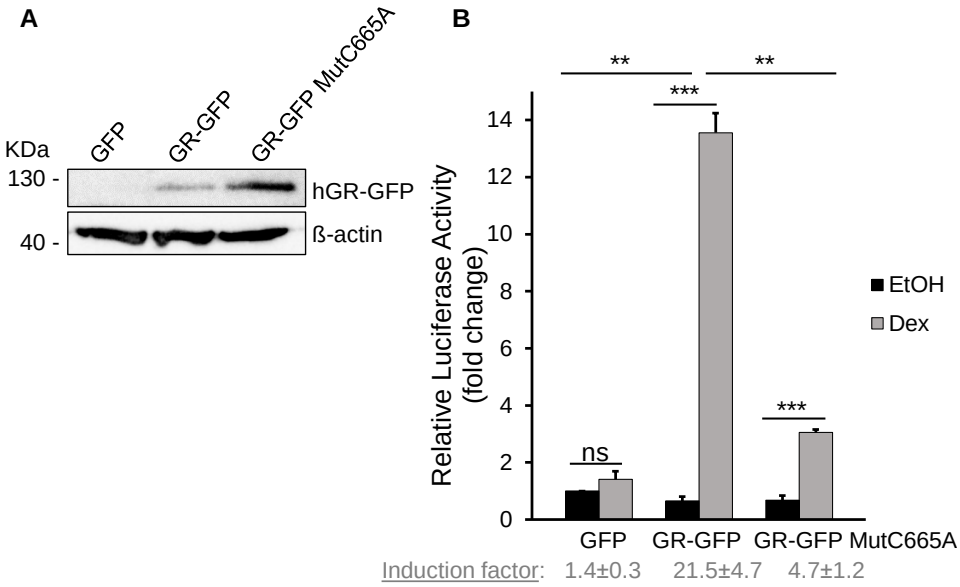


**Figure 3.11:** Effect of GR C665A mutation on transactivation by the GR in COS-7 cells

**(A)** COS-7 cells ($2x10^6$) were transiently transfected with expression vectors coding for GFP alone, GR-GFP or GR-GFP MutC665A and the cells were lysed and immunoblotted. Specific anti-human GR antibodies were used to detect the transfected receptor (h GR-GFP). Anti-ß-actin antibodies were used to demonstrate equal protein loading. **(B)** Luciferase reporter gene assay was carried out to assess the impact of GR MutC665A compared to the wild-type GR on transactivation. COS-7 cells were co-transfected with murine mammary tumor virus-luciferase (MMTV-luc) reporter gene construct and a plasmid expressing Renilla luciferase enzyme as a control for transfection efficiency. The cells were treated with vehicle (0.001% EtOH – black bars) or $10^{-7}$ M Dex (gray bars) for 24 h and luciferase activity was measured. Shown are the bar charts of the results expressed in fold change relative to the vehicle control of GFP. *Data are means ± SEM (n=3). ** P≤0.01; *** P≤0.001; ns=not significant.*

### 3.5.2 Effect of the mutation C665A on the membrane localization of the GR

The localization at the plasma membrane of the mutant C665A GR was analyzed in comparison to the wild-type receptor using a micro-patterned allergen array tool.
Micro-patterned surfaces based, for example, on dip-pen nanolithography (DPN) deposition of allergens in a phospholipids mixture exist already as effective tools for research on mast cells activation and its regulation by GCs [100, 121]. However, their low stability due the non-covalent attachment of the allergens to the surface, makes their application with cells in solution difficult. In this study, another technique for the surface immobilization of the allergens was adopted that would increase the stability of the pattern and make it easier to use. RBL-2H3 mast cells stably expressing GR-GFP and GR-GFP MutC665A were previously sensitized against the target allergen (DNP) and then loaded in solution onto micro-patterned allergen arrays printed by Kumar R. on a glass surface by polymer pen lithography (PPL) [270, 284]. This method was devised by Kumar and Bonicelli *et al.* (2016) and involves the use of click-chemistry to covalently bind the allergens to a surface and, more importantly, to activate the sensitized mast cells [270]. The interaction between the allergens and the glass slide is strong because it exploits the covalent copper (I) catalyzed cycloaddition (CuAAC) reaction between the allergens themselves (DNP-azide) and alkyne groups of the glass slide surface that was previously modified by silanization (see paragraph 2.2.5.2 of the method section). The CuAAC has been shown to be compatible with protein binding and cell culture applications [271, 285–287] and this method allows to visualize, in a single cell approach, events at the IgE receptor-allergen interface.
To perform the experiment, RBL-2H3 mast cells which stably expressed GR-GFP or GR-GFP MutC665A were sensitized for 2 h with Alexa647 labeled anti-DNP IgE, harvested ($1x10^6$ cells/ml) and loaded onto the micro-patterned glass slide. The cells were then allowed to settle on the DNP-azide pattern for 5 or 15 min and after this time they were fixed with 3.7% paraformaldehyde for 20 min. The recruitment of the receptor in proximity of the labeled FcεRI-IgE-DNP interface was investigated using fluorescence inverted microscopy. First, the samples were imaged in Cy5 channel (650 nm laser) to identify the cells that cross-reacted with the DNP pattern. This interaction was visible as purple dots due to the binding of Alexa647 IgE to the DNP-azide arrays. Second, the samples were imaged in EGFP channel (488 nm laser) to detect the positively transfected cells (green) and to determine whether there was co-localization of the fluorescent GR with the IgE receptor-DNP pattern complex.
The results in Figure 3.12 A show accumulation of both the GR-GFP and GR-GFP MutC665A (green dots – GFP panel) in the proximity of the IgE receptor-DNP interface (purple dots – Cy5 panel) after 5 and 15 min following IgE receptor crosslinking. The quantification of the results, shown in Figure 3.12 B, was obtained counting the cells of at least 14 images. The percentage of cells showing accumulation of the fluorescent GR (green dots) was calculated relative to the total number of positively transfected cells (green cells) that also showed Alexa647 IgE-DNP interaction visible as purple dots. The mutation C665A of the GR caused a slight impairment of the membrane recruitment of the receptor. Thus, the cysteine 665 of the hGR putative palmitoylation motif is not required for the localization of the receptor at the plasma membrane.

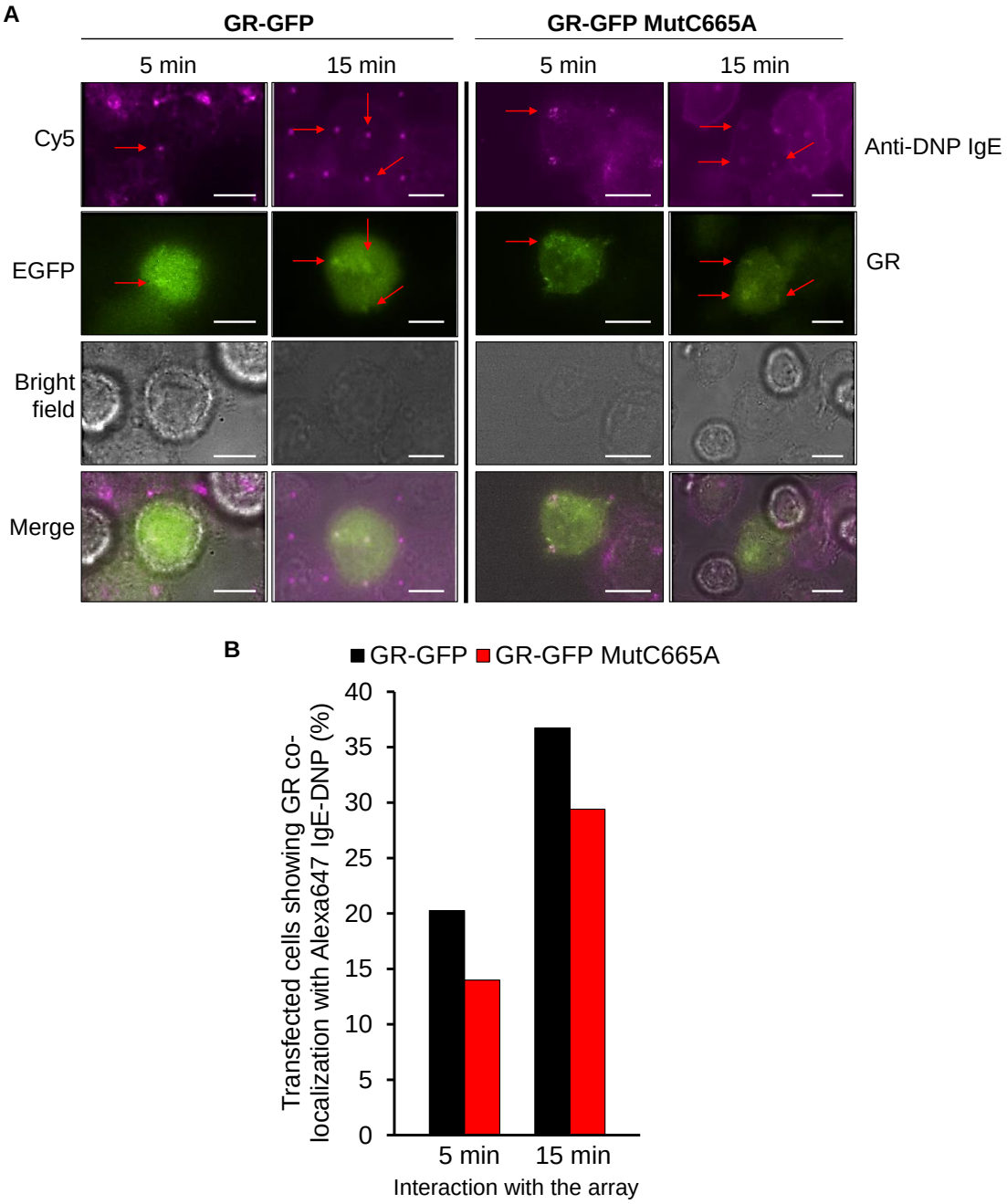


**Figure 3.12:** Effect of C665A mutation on GR membrane localization

RBL-2H3 mast cells stably expressing GR-GFP and GR-GFP MutC665A were sensitized with Alexa647 labeled anti-DNP IgE (0.5 µg/ml) for 2 h, harvested in Tyrode's buffer ($1x10^6$ cells/ml) and then loaded on the micro-patterned glass slide and allowed to settle on the DNP array for 5 and 15 min. After this time, the cells attached to the pattern were fixed with 3.7% paraformaldehyde and images were captured at 60X magnification in EGFP (488 nm laser – green emission light), Cy5 (650 nm laser – purple emission light) and bright field channel using an inverted fluorescence microscope. **(A)** The membrane localization of GR-GFP (left panels) and GR-GFP MutC665A (right panels) is shown at 5 and 15 min. In the upper panel labeled IgE-DNP interaction is visible as purple dots that correspond to the DNP arrays and is indicated by red arrows. In the GFP channel (green), the recruitment of fluorescent GR to the FcεRI-IgE-DNP interface is visible as green dots and is indicated by red arrows. The bright field panel shows the shape of the cells by simple optical illumination and the lower panel shows a merge of the previous channels. The scale bars are equal and correspond to 10 µm. **(B)** Quantification of the experiment in A. The bars represent the percentage of cells showing GR accumulation (green dots) relative to the total number of transfected cells that showed IgE-DNP interaction (purple dots). Data are shown at 5 and 15 min upon FcεRI crosslinking in GR-GFP (black bars) and GR-GFP MutC665A (red bars) transfected cells. Data were calculated from 14 images per time point.

## 3.6 Dynamics of the GR at the plasma membrane

To determine the dynamics of the recruitment of the GR to the plasma membrane, as a further indication and characterization of the involvement of the receptor in the rapid action of GCs, single molecule tracking experiments were performed with photo-activated localization microscopy (PALM) using a GR construct fused to the fluorescent protein mEos2 (mEos2-GR). The mEos2 is a photo-convertible protein that allows, when fused to a target molecule like GR, to detect and track its movement inside the cell. In this experiment RBL-2H3 mast cells were transiently transfected with the mEos2-GR construct and total internal reflection microscopy (TIRF) was carried out in real time for 15 min. TIRF is a particular type of microscopy characterized by a small penetration thickness of 100 nm considered as the proximity of the cell membrane. This helps live cells imaging of very superficial processes at high resolution [288]. The transfected RBL-2H3 cells were treated with vehicle (EtOH) as the negative control or with $10^{-7}$ M Dex. The cells were otherwise previously sensitized with anti-DNP IgE for 2 h and then activated with DNP-BSA or treated simultaneously with DNP-BSA and $10^{-7}$ M Dex. All these treatments were performed under the microscope heated at 37°C and the cells were immediately imaged in real time for 15 min.
In the control experiment (vehicle treated cells – black) the GR manifested high motility at the proximity of the plasma membrane described by a diffusion coefficient in a range (0.1-1 $\mu m^2$/sec) that characterizes the free random movement of particles in a fluid (Brownian motion). The movement of the receptor was, however, more confined and slowed down upon treatment of the cells with $10^{-7}$ M Dex (red) as well as upon activation of the cells through FcεRI receptor crosslinking (green) or when the cells were both activated and treated with Dex (blue). The diffusion coefficient upon any of these treatments was shifted to the left ($10^{-3}$ - $10^{-2}$ $\mu m^2$/sec) compared to the vehicle control (Figure 3.13).

This result demonstrates dynamic changes in the motility of the GR in proximity of the plasma membrane indicative of possible interaction of the receptor with cytoplasmic proteins or membrane components, and this could account for its rapid effects.

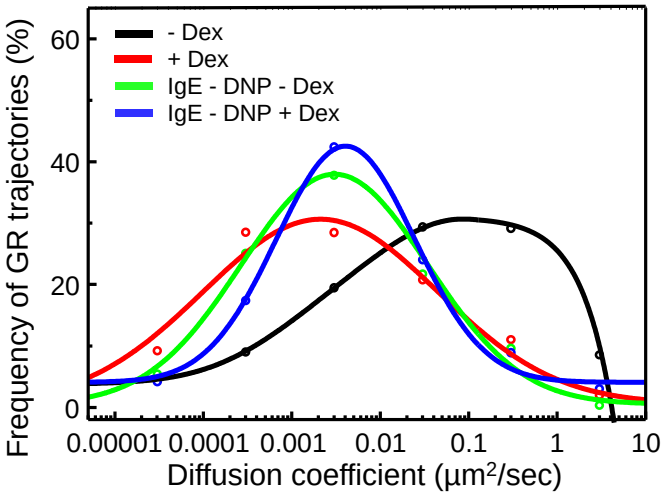


**Figure 3.13:** Single molecule tracking of GR at the plasma membrane

Trend line graph of single GR molecule tracking experiment in RBL-2H3 mast cells. In both charts the frequency of the GR trajectories (%) is plotted over the diffusion coefficient ($\mu m^2$/sec) of the GR molecules. The dynamics of mEos2-GR molecules at the plasma membrane was investigated using TIRF microscopy. RBL-2H3 mast cells were transiently transfected with mEos2-GR 48 h prior to the experiment. The transfected cells were then treated with vehicle (0.001% EtOH – black) or $10^{-7}$ M Dex (red). The transfected cells were otherwise previously sensitized with anti-DNP IgE (0.5 µg/ml) for 2 h and activated with DNP-BSA (500 ng/ml – green) or treated simultaneously with DNP-BSA (500 ng/ml) and $10^{-7}$ M Dex (blue).

# 4 DISCUSSION

GCs are steroid hormones commonly used in the treatment of allergic, autoimmune and chronic inflammatory conditions [11]. Although they are the most potent drugs for the relief of the symptoms of such pathologies, their prolonged use leads to several detrimental effects [12–14]. In order to help the development of new drugs with a better benefit-risk ratio than the conventional ones, the mechanisms of action of GCs was investigated in this study. Classically, the hormone enters the cell and activates the GR which translocates into the nucleus to regulate the expression of hormone responsive genes [35]. This process requires at least 30 min to occur. Alternatively, GCs can act within seconds to minutes via an unknown mechanism whose comprehension may be a turning point toward the goal to identify novel therapeutic targets [289]. A compound selectively directed against these targets might constitute a novel anti-inflammatory drug with little adverse effects. The aim of this research work was to investigate the rapid action of GCs in mast cells, as they play a central role in allergy and anaphylaxis which are disorders that are known to be rapidly suppressed by GC treatment.

In the study presented here a rapid (10 - 15 min) inhibitory effect of the synthetic GC, dexamethasone (Dex) on degranulation of mouse mast cells stimulated via the IgE receptor was observed. This effect of the hormone was mediated by the classical GR since it was inhibited by the GR antagonist RU486. Furthermore, the rapid action of the hormone was lost in mast cells derived from GR knocked-out mice. The mechanism by which the GR mediates this rapid effect of GC in activated mast cells was further investigated. The classical action of GCs that requires translocation of the GR into the nucleus was excluded as a possible mechanism of mediating this rapid effect. The long time that it takes to occur is incompatible with the rapid effect of the hormone observed in this study. Rather, the localization of the receptor at the plasma cell membrane was more likely to be the mechanism involved. Many controversies exist on whether a novel GR is expressed as a membrane-bound protein (mGR) [105–109] or if the classical cytosolic GR is recruited to the plasma membrane to rapidly mediate the function of the hormone [100]. While the former hypothesis has not yet been directly demonstrated, the latter was validated by Oppong et al. (2014) in RBL-2H3 mast cells using live-cell fluorescence microscopy approach [100]. In the work presented here, the recruitment of the classical receptor to the plasma membrane was also validated. To understand the mechanisms involved, the motif “yl**c**mktllls” and in particular the cysteine at position 665 of the human GR was investigated as possible signal for the membrane localization of the receptor. This motif is common to the nuclear receptors and the homologous cysteine at position 447 of the human estrogen receptor, for example, is known to be an

important site of interaction of this receptor with the plasma membrane via palmitoylation. In this study, sensitized RBL-2H3 mast cells stably expressing a mutant C665A GR fused to the green fluorescent protein GFP, were allowed to interact with an allergen array for 5 and 15 min and then the localization of the GR-GFP MutC665A was visualized by fluorescence microscopy. The mutation of the Cys665 to an alanine impaired the ability of the receptor to localize at the plasma membrane. In addition, single molecule tracking of the hGR in the proximity of the plasma membrane was also carried out in RBL-2H3 mast cells. The results, obtained over 15 min by total internal reflection microscopy (TIRF) imaging, revealed a reduced mobility of the receptor following treatment of the cells with GC. A reduced motion of the GR was also observed upon activation of the cells by IgE crosslinking alone or in combination with GC. These results suggest that the GR may be directly recruited to plasma cell membrane or may be trafficked by membrane bound proteins to exert its rapid action.

## 4.1 Rapid action of glucocorticoids in BMMCs and PCMCs

For a long time the rapid action of GCs has been under debate. However, in the last decade it has been accepted by the research community and further investigated. In contrast to neuronal cells [290, 291], airway cells [94], macrophages [124, 292] and T cells [130, 293, 294] which have often been used as models to investigate the rapid mechanism of action of GCs, very little is known about it in mast cells.

### 4.1.1 Effects of Dex on the degranulation of mast cells

Degranulation of antigen activated mast cells is the first event responsible for the symptoms of allergy and anaphylaxis. It is also the first and immediate way for mast cells to release inflammatory mediators such as histamine, serotonin, heparin and enzymes. In the present study, rapid inhibitory effects of Dex on the release of ß-hexosaminidase, a marker for mast cell degranulation, were observed at 10 and 15 min after treatment of mouse bone marrow (BMMCs) and mouse peritoneal (PCMCs) mast cells with the hormone respectively. This result was consistent with the findings of Liu *et al*. (2007) [122] and Zhou *et al.* (2008) [123] that showed a rapid (10 - 15 min) GC-mediated inhibition of degranulation of lung tissue mast cells in guinea pigs with allergic asthma and also GC-mediated inhibition of histamine release from activated RBL-2H3 mast cells and rat peritoneal mast cells. In the work presented here, moreover, the effects of Dex were only transiently observed at 10 - 15 min and were again seen after 1h. This suggests a dual function of the hormone: a possible preparatory and immediate effect of GCs (10 - 15 min) that may be required for the subsequent classical genomic action that occurs at a later time point (1h). This hypothesis is supported by earlier studies of the hormone were similar observations were made [83–85]. However, in contrast to the reports of Liu *et al.* (2007) and Zhou *et al.* (2008) that showed that the effects of GC were not mediated by GR since they were not inhibited by the GR antagonist RU486, the effects

seen in this work in BMMCs and PCMCs were dependent on the classical GR. They were completely abolished by the RU486 inhibitor. The discrepancy between the study presented here and that reported by Liu *et al.* (2007) and Zhou *et al.* (2008) is possibly due to the different type of GCs used to perform the experiments. While these authors used the natural GC corticosterone (CORT) in their studies, in this work the synthetic GC Dex was used to treat the mast cells. It is known that CORT has a strong mineralocorticoid activity exerted through its binding to the mineralocorticoid receptor (MR) [295]. On the contrary Dex has almost no mineralocorticoid activity and it preferentially binds to the GR [296]. Since RU486 exerts its inhibitory action on the GR, but not on the MR [297], it is possible that the rapid effects of corticosterone on mast cells degranulation seen by Liu *et al.* (2007) and Zhou *et al.* (2008) were mostly mediated by the MR and less by the GR, and therefore they resulted insensitive to the GR inhibitor.

The dependency of the rapid GCs effects on the classical GR found in the present study, was definitely confirmed by the results obtained in GR knocked-out PCMCs. The lack of expression of the receptor in these cells prevented the Dex from exerting its early as well as its late inhibitory effects on the degranulation.

### 4.1.2 Effects of Dex on the MAPKs phosphorylation

The modulation of protein kinase activities has often been used as a parameter to investigate the rapid action of GCs in various cell types. For example, Erk1/2 which is a downstream mitogen-activated protein kinase (MAPK) of the IgE-receptor signaling pathway in allergen-activated mast cells, is one of the most studied kinases, whose activity has recently been reported by Oppong *et al.* (2014) to be rapidly (5 - 9 min) up-regulated by GCs in activated RBL-2H3 mast cells [100]. In the study presented here, the same trend of GC up-regulation of Erk1/2 phosphorylation found by Oppong *et al.* (2014) was observed in activated BMMCs between 5 and 10 min. However, due to its high variability from experiment to experiment this effect of the hormone was not statistically significant and therefore not further investigated. At later time points, on the contrary, this research work could detect, for the first time in mast cells, constant inhibitory effects of Dex on Erk1/2, JNK and, to a lesser extent, on p38 phosphorylation already at 30 min or 60 min of hormone treatment in activated BMMCs and PCMCs respectively. In other studies, effects of GCs on the three MAPKs phosphorylation were observed starting from 8h for Erk1/2 or 4h for p38 [260] in BMMCs or starting from 6h for JNK in RBL-2H3 mast cells [256] and were all mediated by genomic mechanism of action of GCs. In the case of the effects seen in the present study, their timing at 30 and 60 min could account for a rapid genomic action of the hormone or could be mediated by a non-genomic mechanism of action of GCs. To validate this observation (30 - 60 min), the protein synthesis inhibitor cycloheximide (CHX) was applied to the cells and the effect of Dex on phosphorylation of Erk1/2 was analyzed. CHX prevented the hormone from inhibiting Erk1/2 phosphorylation from 30 and 60 min and therefore these effects required the synthesis of proteins to occur. They were most probably driven by rapid genomic mechanism of action of Dex. In addition,

the treatment of the cells with the inhibitor RU486 or the GR knocking-out of mast cells, prevented the inhibitory effects of Dex on Erk1/2. Therefore, the GR is required to mediate the effects of Dex. Kassel *et al.* (2002) demonstrated that treatment of RBL-2H3 mast cells with the Erk1/2 inhibitor, U0126, significantly reduced the percentage of ß-hexosaminidase released upon allergen activation [298]. This finding suggests a possible cross-talk between the Erk1/2 activation pathway and the degranulation pathway which could explain the results found in this study. The down-regulation of Erk1/2 phosphorylation at 30 - 60 min may be involved in the inhibition of ß-hexosaminidase release from 1h.

## 4.2 The involvement of the glucocorticoid receptor in the rapid action of glucocorticoids and its membrane localization

To investigate the involvement of the GR in the rapid action of GCs, the GR inhibitor RU486 is often used. The results in the field are quite contradictory and some authors believe in a rapid action of GCs mediated by a membrane-bound GR (mGR) which is different from the classical one and this is the reason why the inhibitor RU486 does not have any effect on it. On the other hand, in studies like the one presented here, the rapid effects of GCs are sensitive to the RU486 inhibitor and therefore they are thought to be mediated by the classical GR which translocates to the plasma membrane. It should be pointed out that discrepancies between different research works are also possibly due to differences between the cell types used to carry out the experiments, the type of GC used, and also the experimental conditions. However, the most straightforward way to validate the role of a protein like GR in a physiological process, is the generation of an organism that has been knocked-out for this gene of interest [299]. In the case of the GR, it is not possible to generate a total knock-out of the protein due to the post-natal mortality of the animals as a result of respiratory failure and impaired liver gluconeogenesis capacity [300]. Therefore, in the present study the GR was specifically knocked-out in PCMCs via Cre/loxP recombination system. Investigating the effects of Dex on mast cells which did not express the receptor, it was demonstrated that this protein is fundamental to mediate the early effects of the hormone on their degranulation and Erk1/2 phosphorylation.

While the effects of GC on Erk1/2 activation at 30 - 60 min were clearly mediated by genomic mechanism of action of the hormone, as demonstrated by CHX administration, the effects seen at 10 - 15 min on the degranulation were far too rapid to be due to changes at the genomic level. Rather, these last events were possibly mediated by a membrane localization of the GR, as postulated by other authors [100]. Preliminary evidence of the association of the classical GR with the plasma membrane were observed by Matthew *et al.* (2008) in lung epithelial cells via fractionation and co-immunoprecipitation methods. They showed that the GR was associated with a membrane lipid raft protein called caveolin-1 [120]. Further confirmation that the classical cytosolic receptor can be recruited to the plasma membrane was shown by

Oppong *et al.* in 2014 via live-cell microscopy. The GR was rapidly (within 15 min) recruited to the plasma membrane of activated mast cells (even in the absence of the hormone) [100]. At this location the receptor may readily bind the hormone to mediate its early effects.

## 4.3 Mechanisms of GR membrane localization

Although evidence in mast cells suggests that the classical GR can localize at the plasma membrane [100], the exact mechanism responsible for its translocation remains unknown. In the present study, the putative palmitoylation site Cys665 at the hormone binding domain of the hGR was investigated as possible anchor of the GR to the plasma membrane.

### 4.3.1 Nuclear localization and transactivation activity of the GR MutC665A-GFP

Since Cys665 is located at the hormone binding domain of the receptor, it was first investigated in this study whether the mutation affected the classical GR response to hormone binding. The results showed that the mutated GR could translocate into the nucleus and also promote transactivation, but these functions were significantly reduced compared to the wild-type receptor. The peculiar localization of the cysteine, corresponding to helix 8 of the hormone-binding domain, may be responsible for altering the ligand binding and that could explain the decreased receptor function. However, the mechanism by which mutation in helix 8 could impair ligand binding is not clear, since this helix is not part of the binding pocket [301, 302]. More likely, Cys665 plays a role in maintaining the correct receptor conformation in order to allow the interaction of the ligand with its binding pocket, however this was not further explored in this study. These results are consistent with the finding of Deng *et al.* (2015) that showed that a homologous mutation in the rat GR (C683A) did not affect the nuclear translocation nor the transactivation function of the receptor in the hypothalamic cell line 4B [303].

### 4.3.2 Localization of GR MutC665A-GFP at the plasma cellular membrane

The investigation of the site of localization of the mutated (C665A) GR-GFP in stably transfected RBL-2H3 mast cells was carried out in the present study using micro-patterned arrays of allergen generated on a glass surface by polymer-pen lithography (PPL). Over the years, several techniques have been developed that allow the patterning of molecules on a surface and many of them are based on the conventional micro-contact printing, where a silicon stamp acts as a master for the printing of the pattern of the desired molecules [304]. Alternatively, automated printing systems such as dip-pen nanolithography (DPN) exist, which are characterized by the release on the surface of little and ordered drops of ink containing the substance of interest [100, 121]. This is, for example, the tool used by Oppong *et al.* (2014) to generate arrays of allergen in a phospholipid mixture that allowed the visualization of the GR-GFP recruitment to the plasma membrane [100]. Advantage of the DPN is to have the allergen

deposited on the surface in a fluidic state which is optimal to mimic the physiological condition where the allergen and the IgE-receptor are internalized by the activated cells. However, a major disadvantage of this system is that the array is not covalently attached to the surface and therefore it is not stable, but easily washable out when the cells are loaded in solution to perform the experiment. The PPL technique used in this study can be viewed as a hybrid technique of DPN and conventional micro-contact printing via click-chemistry that was developed and successfully tested for mast cells activation in parallel to this research project by Kumar and Bonicelli *et al.* (2016) [270]. Using PPL the molecules of allergen azide-conjugated were covalently bound to a modified (silanized) surface and therefore the pattern was very stable during the experiment. In this study, incubation of GR-GFP MutC665A and GR-GFP transfected RBL-2H3 mast cells with the allergen arrays led to the membrane recruitment of both the receptors with a slight decrease for the mutant GR. Therefore, the Cys665 is not required for the membrane recruitment of the GR which does not undergo palmitoylation, as also recently demonstrated by Deng *et al.* (2015) in hypothalamic cell line 4B [303].

## 4.4 Dynamics of the GR at the plasma membrane

In the work presented here, photo-activated localization microscopy (PALM) was used to perform single molecule tracking [305] to investigate the dynamics of the wild-type GR at the plasma membrane. For this purpose, TIRF microscopy was utilized to focus within the thickness of 100 nm into the single cells, corresponding to the cell membrane. The results obtained from 15 min of TIRF microscopy revealed that the treatment of the cells with GCs, IgE-DNP or a combination of GCs and IgE-DNP leads to a reduction of the mobility of the receptor compared to the control (vehicle). This means that after any of the treatments, the receptors located in the proximity of the plasma membrane, move slower and along shorter trajectories. Reason for this can be conformational changes of the receptors following the treatments, for example due to their phosphorylation mediated by MAPKs activation. This may impair their mobility and also may facilitate their interaction with cytoplasmic molecules or the plasma membrane. The PALM results presented here were consistent with recently findings where fluorescence recovery after photo-bleaching (FRAP) was also used to investigate the motility of the receptor in the proximity of the plasma membrane of RBL-2H3 mast cells. This technique consists of the bleaching of the fluorescent GR molecules with short, but very intense laser light and then calculation of the time needed to the molecules to recover the fluorescence intensity. It was shown that activated mast cells needed a longer time to recover the fluorescent intensity than the non-activated cells and therefore this also suggested a possible interaction of the receptor with the plasma membrane or cytoplasmic proteins [100].

## 4.5 Model for the rapid action of glucocorticoids in mast cells

Upon activation of the cell, the GR is recruited to the plasma membrane through an unknown mechanism that does not involve palmitoylation. Probably, the receptor is phosphorylated by signaling molecules among which the MAPKs and this helps its interaction with other cytoplasmic proteins such as Lyn, Fyn, Syk or PLCγ which are recruited to the IgE-receptor to initiate the signaling pathway. At the membrane, GR is located in proximity of the IgE-receptor and here it mediates the rapid (10 - 15 min) inhibitory effects of GCs on the degranulation of the cells. Phosphorylation of the GR also helps it to be involved in genomic action of GCs that could be detected earliest at 30 - 60 min on inhibition of MAPKs phosphorylation and 60 min on inhibition of the degranulation.

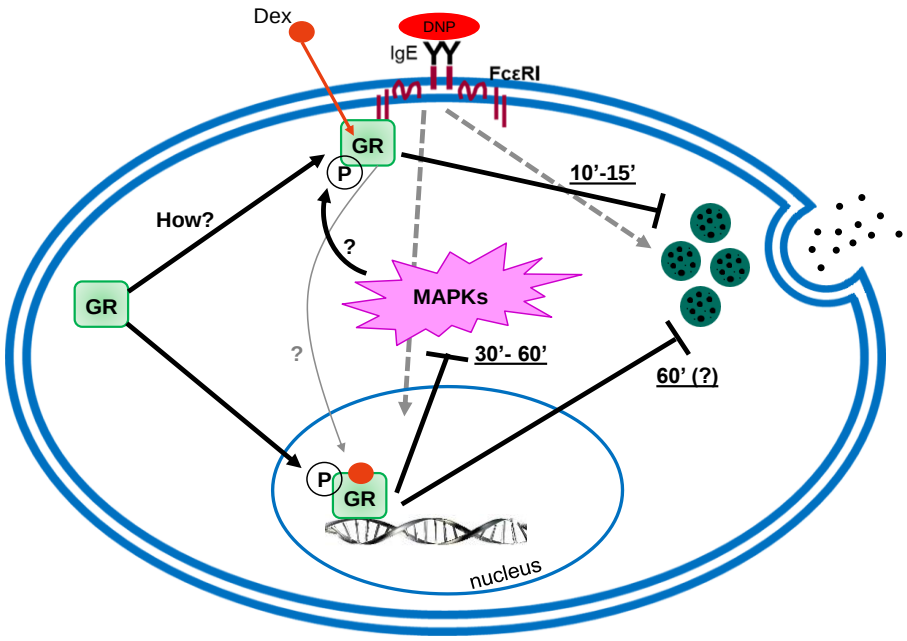


**Figure 4.1:** Model for the rapid action of GCs in mast cells

The GR is located at the plasma membrane via unknown mechanism. Here it mediates the rapid action of GCs on degranulation. From its location in the nucleus, the receptor mediates rapid 30-60 min effects of GCs on MAPKs phosphorylation and probably also on degranulation.

## 4.6 Conclusion

In conclusion, this research project demonstrated rapid inhibitory effects of Dex on the degranulation of allergen activated BMMCs and PCMCs that occur between 10 to 15 min. These effects are mediated by the classical GR, as demonstrated by the absence of Dex effects in GR knock-out mast cells. Moreover, the GR exerts its function most likely through its recruitment to the plasma membrane as soon as the cells are activated by the binding of the allergen to the FcεRI receptor. The mode by which the GR is recruited to the plasma membrane

or how it mediates the rapid hormone effects remains unclear, but it was observed here that the Cys665 is not involved. Single molecule tracking of the GR molecules in the proximity of the plasma membrane suggested a possible interaction of the receptor with the plasma membrane or with other proteins recruited to the membrane which needs to be further investigated.

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CE L’HO FATTA!

# Curriculum Vitae

| | |
|---|---|
| **Name:** | Alice Bonicelli |
| **Date of birth:** | 10th September 1985 |
| **Place of birth:** | Bologna (Italy) |

**University Education**

| | |
|---|---|
| 06/2013 to 05/2016: | **PhD student** at Karlsruhe Institute of Technology (KIT) Institute of Toxicology and Genetics (ITG) |
| 09/2004 to 02/2012: | **Master's Degree** in Veterinary Medicine at Bologna University (Italy) |

**Practical Education**

| | |
|---|---|
| 04/2012 to 10/2012: | **Centro Veterinario Bolognese S.r.l. (Bologna - Italy)** – 6 months internship in the clinic for small animals |
| 12/2011 to 10/2012: | **Ambulatorio Veterinario Anzola (Bologna - Italy)** – practical experience in the clinic for small animals |
| 12/2009 to 01/2013: | **Ambulatorio Veterinario Associato Baldi-Visentin (Bologna - Italy)** – practical experience in the clinic for small animals |
| 10/2007 to 12/2010: | **Bologna University (Italy)** – practical experience in the oncological surgery department |

**School**

| | |
|---|---|
| 09/1999 to 06/2004: | **Scientific High School (Bologna - Italy)** |

**Presentations and Posters**

| | |
|---|---|
| 09/2016 | “Rapid action of glucocorticoids in mast cells” Institute Seminar (talk) |
| 02/2016 | “Rapid action of glucocorticoids in mast cells” PhD seminar (talk) |
| 07/2015 | “Rapid non-genomic action of glucocorticoids in mast cells” BIFTM retreat (talk) |
| 07/2015 | “Rapid non-genomic action of glucocorticoids in mast cells” Institute Seminar (talk) |
| 12/2014 | “Rapid non-genomic action of glucocorticoids in mast cells” International mast cell and basophil meeting in Munich (poster presentation) |
| 09/2014 | “Rapid non-genomic action of glucocorticoids in mast cells” BIFTM retreat (poster presentation) |
| 07/2014 | “Rapid non-genomic action of glucocorticoids in mast cells” Institute Seminar (talk) |

**Publications**

**“Click-Chemistry based allergen arrays generated by polymer pen lithography for mast cell activation studies”** Ravi Kumar*, Alice Bonicelli*, Sylwia Sekula-Neuner, Andrew C. B. Cato, Michael Hirtz and Harald Fuchs (*Small, vol. 12, no. 38, pp. 5330–5338, 2016*)

*equal contribution

**Teaching**

Signaltransduktion und Genregulation (F2: Practical course)

Biologisches Methodenpraktikum

Schnupperpraktika