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Reynod A. Panettieri
Rutgers Institute for Translational Medicine & Science

Dedmer Schaafsma
Science Impact, Winnipeg

Yassine Amrani
Leicester Biomedical Research Center Respiratory

Cynthia Koziol-White
Rutgers Institute for Translational Medicine and Science

Rennolds S. Ostrom
Chapman University, rostrom@chapman.edu

See next page for additional authors

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Authors
Reynod A. Panettieri, Dedmer Schaafsma, Yassine Amrani, Cynthia Koziol-White, Rennolds S. Ostrom, and Omar Tliba

NON-GENOMIC EFFECTS OF GLUCOCORTICOIDS: AN UPDATED VIEW

Reynold A. Panettieri[1], Rutgers Institute for Translational Medicine & Science, Robert Wood Johnson School of Medicine, New Brunswick, USA.

Dedmer Schaafsma[2], Science Impact, Winnipeg, MB, R3L 2S9, Canada.

Yassine Amrani[3], Institute for Lung Health, Leicester Biomedical Research Center Respiratory, Leicester, U.K.

Cynthia Koziol-White[1], Rutgers Institute for Translational Medicine & Science, Robert Wood Johnson School of Medicine, New Brunswick, NJ, USA.

Rennolds Ostrom[4], Department of Biomedical and Pharmaceutical Sciences, Chapman University School of Pharmacy, Irvine, CA, USA.

Omar Tliba[5], Department of Biomedical Sciences, College of Veterinary Medicine, Long Island University, Brookville, NY, USA.

*Correspondence: omar.tliba@liu.edu (O. Tliba)

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Abstract

Glucocorticoid (GC) anti-inflammatory effects generally require a prolonged onset of action and involve genomic processes. Because of the rapidity of some of GC effects, however, the concept that non-genomic actions may contribute to GC mechanisms of action has arisen. While the mechanisms have not been completely elucidated, the non-genomic effects may play a role in the management of inflammatory diseases. For instance, we recently reported that GC “rapidly”

enhanced the effects of bronchodilators, agents used in the treatment of allergic asthma. In this review, we will discuss i) the non-genomic effects of GCs on pathways relevant to the pathogenesis of inflammatory diseases and ii) the putative role of membrane GC receptor. Since GC side effects are often considered to be generated through its genomic actions, understanding GC non-genomic effects will help design GCs with a better therapeutic index.

Mechanism of action of glucocorticoids (GC).

GCs primarily mediate their effects by activating the ubiquitously expressed intracellular **GC receptor (GR)** (see Glossary) [1]. In its inactive state, the GR resides in the cytoplasm and, upon ligand activation, translocates to the cell nucleus to interact with GC response elements (GREs) thereby producing **genomic effects** that alter protein expression. Interestingly, evidence suggests that GCs also manifest almost immediate **non-genomic actions** on several signaling processes [2]. GC non-genomic effects involve non-specific interactions with the cell membrane, or specific interactions with cytosolic GRs (cGR) or membrane-bound GRs (mGR) (Table 1). This report summarizes the current knowledge on non-genomic effects of GCs, with a focus on GR-mediated events and GR-associated signaling pathways. Where appropriate, potential links to inflammatory diseases will be highlighted in the main text and their potential impact will be discussed in Box 1 and 2.

GCs exert rapid effects on levels of intracellular calcium.

Studies suggest that GC rapidly (within seconds) modulates basal intracellular calcium levels and agonist-induced **calcium mobilization** (Tables 2 & 3).

Effects of GCs on intracellular calcium homeostasis. GCs can increase or decrease cytosolic calcium depending on the cell type. Evidence from non-immune cells, such as primary or immortalized human bronchial epithelial cells, consistently demonstrate that acute exposure to GC, and to a lesser extent to the mineralocorticoid (MC), aldosterone, reduces basal $[Ca^{2+}]i$ [3, 4]. Similarly, in rat thymocytes [5] and mouse neuroblastoma cells [6] $[Ca^{2+}]i$ decreased following acute exposure to GC, and in cichlid fish pituitary cells cortisol inhibited $[Ca^{2+}]i$ and reduced prolactin secretion [7]. However, in immune cells, it is unclear if GCs genuinely exert non-genomic effects on basal $[Ca^{2+}]i$. For example, while acute exposure to GC was reported to decrease $[Ca^{2+}]i$ in leukocytes, these leukocytes were obtained from donors who were treated with oral prednisolone for 7 days [8], potentially confounding the results of the study. Similarly, studies in human lymphoblasts show that cortisol markedly reduced basal $[Ca^{2+}]i$ only after 48 hrs of treatment [9]. These data argue against a role for non-genomic effects of GC in altering basal $[Ca^{2+}]i$ in immune cells.

With regard to the lungs, evidence supports that a variety of GCs differentially modulate basal $[Ca^{2+}]i$ upon immediate exposure. For instance, the acute inhibitory effects of dexamethasone (within 30 seconds) on basal $[Ca^{2+}]i$ in bronchial epithelial cells were comparable to triamcinolone acetonide and hydrocortisone but not to budesonide [10]. Interestingly, the GR antagonist RU486 and the protein synthesis inhibitor cycloheximide failed to prevent these acute GC effects, suggesting the involvement of GR-independent and non-genomic pathways. These observed effects could be due to the various degrees of lipophilicity among GCs, as well as direct interactions of GCs with the cell membrane [10]. Non-genomic mechanisms have been proposed mostly based on the use of pharmacological inhibitors. Urbach and colleagues found that the rapid GC effects involved pathways regulated by the SERCA type Ca^{2+}-ATPase pump, adenylyl cyclase and protein kinase A (PKA) but not protein kinase C (PKC) [10]. Collectively, these studies show the complexity of mechanisms involved in the rapid, GR-independent effect of GCs on $[Ca^{2+}]i$, which likely occurs through an adenylyl cyclase/PKA mediated stimulation of a thapsigargin sensitive Ca^{2+}-ATPase [10].

Conversely, acute stimulatory effects of GC on basal calcium levels have been documented. A brief exposure to GC can increase $[Ca^{2+}]i$ in several cell types. For example, in mouse cortical collecting duct cells, dexamethasone and aldosterone increased $[Ca^{2+}]i$. Interestingly, the effect of aldosterone was mediated by a non-genomic activation of PKC pathway as evidenced by the abolishment of its effect on basal $[Ca^{2+}]i$ in the presence of the PKC inhibitor, chelerythrine chloride, but not the mRNA synthesis inhibitor, actinomycin D [11]. Similarly, in rat vascular smooth muscle cells, GCs rapidly increased $[Ca^{2+}]i$ [12] potentially through GC-mediated increases in inositol 1,4,5-triphosphate (IP3) levels associated with the translocation of the calcium- and lipid-dependent PKC from the cytosolic to the membranous compartment [13]. In these cells, while the administration of epinephrine by itself had little effect on IP3 levels, epinephrine potentiated the rapid response induced by cortisol [13]. Collectively, these findings highlight a role of PKC in the rapid increase of basal $[Ca^{2+}]i$ by GCs.

Effects of GCs on agonist-induced calcium mobilization. The effects of GCs on agonist-induced calcium mobilization are variable depending on the agonist, the extra-cellular stimuli and the cell type. Evidence suggests that GCs rapidly inhibit, at least partially, the ability of adenosine triphosphate (ATP) to increase $[Ca^{2+}]i$ in some cell types. In human bronchial epithelial cells for

example, 15 min exposure to dexamethasone (1 nM) markedly reduced ATP-induced increases in $[Ca^{2+}]i$. The ATP-induced Ca^{2+} response was independent of extracellular calcium but did involve a Ca^{2+}-mobilization from thapsigargin-sensitive intracellular stores [10]. Similarly, in murine HT4 neuroblastoma cells, acute (5 min) pre-incubation with corticosterone dose-dependently inhibited $[Ca^{2+}]i$ signals induced by ATP [6]. Unlike in human bronchial epithelial cells, the Ca^{2+}-response induced by ATP in these cells relies on Ca^{2+}-influx across the plasma membrane and Ca^{2+}-release from intracellular stores [6]. Inhibition of PKA abrogated the inhibitory action of corticosterone on ATP-induced Ca^{2+}-elevation, whereas little influence was observed with respect to PKC inhibition. Additional studies demonstrated that these GC inhibitory effects were unaffected by GR blockade. These key findings obtained from studies in HT4 cells suggest that GC activates membrane-initiated, non-genomic, PKA-dependent, PKC-independent pathways [6, 14]. In contrast, in rat B103 neuroblastoma cells, the inhibitory effects of corticosterone on serotonin-induced peak $[Ca^{2+}]i$ were found to be PKC-dependent [15]. Together, these studies suggest that the mechanisms mediating the acute non-genomic effects of GC on agonist-evoked calcium mobilization are stimuli and cell type-dependent.

In contrast to human bronchial epithelial and murine HT4 neuroblastoma cells, pretreatment of guinea pig cochlear spiral ganglion neurons (SGN) with dexamethasone (10 min) enhanced ATP-induced Ca^{2+}-mobilization [16]. This effect was prevented in the presence of a GR antagonist and mediated by rapid Ca^{2+}-influx through activation of ionotropic purinergic P2X receptors [16]. Of note, all P2X subtypes are expressed in SGN albeit to different extents [17, 18]. Similarly, in rat hippocampal neurons, pretreatment with corticosterone or dexamethasone for 10-20 min prolonged N-methyl-D-aspartate (NMDA)-induced transient elevation in $[Ca^{2+}]i$ [19]. Importantly, the steroid effect was reversed by the removal of corticosterone indicating that the steroid effect was not due to irreversible impairment of Ca^{2+}-extrusion from the neurons. Thapsigargin and cyclohexamide had little effect on the potentiating effect of corticosterone, excluding the involvement of a thapsigargin sensitive Ca^{2+}-ATPase or *de novo* protein synthesis, respectively. Interestingly, the GC effect was reproduced by the use of a membrane impermeable BSA-conjugated cortisol, suggesting that mGR likely underlies the rapid non-genomic effects of GC [19]. However, canonical genomic actions of GC can also alter Ca^{2+} mobilization. In human lymphoblasts, while cortisol reduced basal $[Ca^{2+}]i$ (as indicated above), Ca^{2+}-mobilization induced

by platelet activating factor (PAF) is enhanced only by chronic treatment (48 hrs) with cortisol [9] (Figure 1).

GCs rapidly modulate skeletal and smooth muscle function.

Several studies have reported variable acute effects of GCs on **muscle reactivity** and tone. The specific example of airway smooth muscle cells in the pathogenesis of inflammatory diseases is highlighted in **Box 1**. In mouse skeletal myotubes (C2C12 immortalized myoblasts), treatment with dexamethasone (for less than 20 min) reduced glucose uptake induced by electrical pulse stimulation (EPS)-mediated contraction, in a Ca^{2+}/calmodulin protein kinase II (CaMKII) and AMP activated protein kinase (AMPK) dependent fashion [20]. The effects were unaffected by blockade of GR (RU486) or inhibition of protein synthesis (cyclohexamide), indicating a rapid non-genomic and GR-independent effect. In another study, cortisol synergized with isoprenaline in reducing tracheal spasms in response to histamine [21]. The spasmolytic effect was fully prevented in the presence of RU486 (implicating a GR-dependent pathway), partially reduced by PKC inhibition, but was unaffected by actinomycin D (excluding *de novo* RNA synthesis) again suggesting a non-genomic, GR-mediated signaling pathway involving PKC [21].

Other studies support a role for GCs in rapidly reducing airway smooth muscle (ASM) tone. Pretreatment with budesonide (within 15 min) suppressed histamine-induced isometric tension in guinea pig tracheal rings and shrinkage in individual tracheal ASM cells; effects that were unaffected by cycloheximide (suggesting non-genomic actions by budesonide) [22]. Unlike the findings by Wang and colleagues [21], these budesonide effects were insensitive to RU486, excluding classic GR involvement [22]. Similarly, in murine ASM cells, exposure to dexamethasone for 10 min decreased basal $[Ca^{2+}]i$ and reduced peak elevations in $[Ca^{2+}]i$ induced by acetylcholine, effects that were insensitive to GR blockade and cycloheximide [23]. Consistently, studies using an *in vivo* guinea pig model of asthma, an established model to study allergen-induced asthmatic reactions and airway hyperresponsiveness [24], revealed a beneficial effect on ovalbumin-induced changes in lung resistance and compliance by acutely inhaled budesonide. The protective effects of budesonide were evident within 10 minutes, suggesting a non-genomic course of action [25]. In summary, GCs have acute spasmolytic actions in ASM that

can require both GR-dependent and -independent pathways, and potentially PKC-mediated signaling.

A recent study in rat vascular smooth muscle cells under conditions of lipopolysaccharide (LPS)-induced septic shock showed that dexamethasone treatment for 10 min promotes norepinephrine (NE)-induced phosphorylation of key proteins associated with contraction [26]. While no significant effect on myosin light chain 20 (MLC20) phosphorylation was observed after exposure to either dexamethasone or NE alone, the combined treatment markedly enhanced phospho-MLC20, an effect that was unaltered by GR blockade with RU486. Interestingly, inhibition of Rho-kinase with Y-27632 completely reversed the potentiating effects of dexamethasone on NE-induced phospho-MLC20. Together, these findings could be of clinical significance and indicate that the impaired vascular response to NE observed in septic shock may be restored by short-term exposure to dexamethasone through non-genomic activation of Rho-kinase activity [26].

GCs exert rapid effects on Reactive Oxygen Species (ROS)/Reactive Nitrogen Species (RNS).

Studies demonstrated a rapid effect of GCs on ROS generation and the involvement of nitric oxide (NO) in mediating some GC effects. An example of the role NO/ROS in the pathogenesis of inflammatory disease is highlighted **in Box 2**. In breast cancer cells, cortisol rapidly increased levels of ROS and RNS (as early as 15 min) and induced DNA damage. The GR antagonist (RU486) blocked the cortisol effect while L-NAME and 1400 W dihydrochloride demonstrated the involvement of nitric oxide synthase (NOS) and inducible (i)NOS, respectively. The pharmacological inhibition of Src by PP2 prevented GC-induced RNS elevation, suggesting the ability of GC to rapidly stimulate Src- and iNOS-dependent release of damaging RNS levels [27].

Rapid effects of GCs on endothelial NOS (eNOS), an important mediator of vascular integrity with anti-inflammatory, anti-ischemic, and anti-atherogenic properties, have been described as well [28-30]. Indeed, the treatment of human vascular endothelial cells with dexamethasone rapidly enhanced (as early as 10 min), in a concentration-dependent manner, eNOS activity, NO-production and NO-dependent vasorelaxation [31]. These GC effects were abrogated

by RU486, PI3-kinase inhibitors wortmannin and LY292002, or L-NAME, but not by the transcriptional inhibitor actinomycin D.

Additional evidence supporting rapid effects of GCs on NOS/NO showed an augmented ATP-induced, NOS-dependent NO release in guinea pig type I spiral ganglion neurons by dexamethasone that was thought to be a consequence of ATP-induced $[Ca^{2+}]i$ [16]. Similarly, GR-mediated increases in $[Ca^{2+}]i$, eNOS phosphorylation, and NO production, were observed in human umbilical vein endothelial cells [32]. Interestingly, NO production increased $[Ca^{2+}]i$ originating from intracellular and extracellular Ca^{2+} sources [32].

The PI3K/Akt pathway is critical in the activation of NO signaling, e.g. phosphorylation of eNOS [33], and the involvement of this pathway in the rapid effects of GCs has been documented [33]. For example, dexamethasone rapidly increased (within 20 min), in a dose-dependent manner, GR-dependent phosphorylation and activation of PI3K as demonstrated by phosphorylation of Akt and glycogen synthase kinase (GSK)-3, indicating that GCs can functionally activate PI3K and downstream targets in human endothelial cells [31]. The potential clinical relevance of these observations was confirmed in two different mouse models of ischemic injury (i.e. transient myocardial ischemia and transient focal cerebral ischemia) where GC exerted rapid protective effects (within 30 min) via GR-dependent activation of PI3K and eNOS pathways as evidenced by the administration of RU486, wortmannin and L-NAME, respectively [31, 32]. Additional studies in COS-7 cells demonstrated a key role for GR in GC-induced activation of the PI3K/Akt pathway. When cells were transfected with a dimerization-defective GR mutant (A458T, a construct that is unable to bind DNA and transactivate GC target genes), acute dexamethasone stimulation still activated the PI3K/Akt pathway [34]. Together, these findings suggest the involvement of a non-transcriptional/non-genomic mechanism in the GR-dependent activation of PI3K/Akt by GCs.

Since NO signaling plays a key role in chronic airway inflammatory diseases, such as asthma and COPD [35], we believe that the cross-talk between GC and NO signaling warrants further investigation to determine whether the rapid effects of GC on NO signaling would be beneficial or detrimental in disease pathogenesis.

GCs exert acute effects on inflammatory and apoptotic pathways.

Evidence shows rapid non-transcriptional actions of GCs on inflammation both in transformed cells and immune cells. In transformed cells, such as A549 adenocarcinoma cells, acute exposure (as early as 1 min) to dexamethasone rapidly inhibited epidermal growth factor (EGF)-induced arachidonic acid (AA) release, an important mediator of inflammation [36]. This inhibitory effect was due to hindering the recruitment of Grb2, p21ras and Raf to the EGF receptor (EGFR) through a GR-dependent (RU486-sensitive) and transcription-independent (actinomycin D-insensitive) mechanism. The inhibition of Grb2 recruitment was accompanied by lipocortin-1 recruitment to EGFR in the cell membrane. Subsequently, lipocortin-1 competitively inhibited Grb2 binding to EGFR, thereby blocking the recruitment of critical signaling molecules necessary for EGF actions [36].

The acute effects of GCs on inflammatory pathways were also observed in immune cells, such as human neutrophils, where acute exposure (5 min) to methylprednisolone or hydrocortisone significantly inhibited N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced neutrophil degranulation, effects that were not prevented by RU486 or cycloheximide treatments, suggesting the involvement of GR-independent and non-genomic pathways [37]. Also, in murine macrophages acutely treated with dexamethasone (30 min), toll like receptor 9 (TLR9)-induced activation of different inflammatory signaling pathways, such as those involving NF-κB and mitogen-activated protein kinases (MAPKs), was dramatically suppressed [38]. Following TLR-9 engagement, IL-1R-associated kinase 1 (IRAK1) is recruited to the cell membrane. A critical step in activating the TLR signaling cascade is the ubiquitination of IRAK1 through its physical interaction with the E3 ligase, β-TrCP. Such ubiquitination and degradation of IRAK1 promotes the trafficking of the "TNFR-associated factor 6 (TRAF6)– TAK1 adaptor proteins (TAB)– Transforming growth factor beta-activated kinase 1 (TAK1)" complex to the cytosol to subsequently induce MAPK and NF-κB activation. Dexamethasone inhibition of IRAK1 ubiquitination did not occur in the presence of RU486 suggesting the involvement of GR-dependent mechanisms [38]. Further investigation of the molecular mechanisms revealed that by physically interacting with IRAK1, GR interferes with the interaction between β-TrCP and IRAK1 thereby impeding its ubiquitination, a critical step in the activation of the TLR9-dependent inflammatory cascade [38].

Rapid GC treatment can also exert pro-inflammatory action in other cell types. For example, in PC12 cells (cell line derived from rat adrenal gland), corticosterone induced rapid

activation (within 15 min) of ERK1/2, p38, and JNK in a PKC-dependent manner [39, 40]. The activation of MAPK pathways following GC treatment appears to be mediated by the putative mGR, since corticosterone-BSA can rapidly (with 15 min) activate all MAPKs [39, 40]. Similarly, in rat vascular smooth muscle cells, dexamethasone either alone or in combination with NE, rapidly (within 10 min) induces ERK1/2 and p38 MAPK activities [26]. Thus, in certain cells, GCs can activate MAPK in a non-genomic manner.

In CCRF-CEM cells, cell line derived from human T-cells (from pediatric ALL patients), sensitivity to acute dexamethasone induced cell death was determined in the presence and absence of phosphodiesterase (PDE) inhibitors [41]. Non-specific PDE and specific PDE4 inhibition reversed steroid resistance and markedly increased sensitivity to dexamethasone. This effect is likely due to increased cAMP levels, consistent with abundant documentation on interactions between GR and cAMP pathways in the induction of apoptosis in lymphoid cells by both [42, 43]. To date, the mechanisms of cAMP-induced apoptosis are unclear, but the presence of GR appears to be required, even in the absence of GCs. For instance, in parental T-cells, elevation of cAMP, with either forskolin or dibutyryl cAMP, induced apoptotic cells death, whereas GR deficient cells were insensitive to the apoptotic effects of cAMP elevation. When GR expression was reconstituted by transfection, not only was GC sensitivity restored, but the sensitivity to cytolytic effects induced by cAMP was promoted as well [42].

The effects of GCs on the mitochondrial control of cell metabolism and apoptosis have been extensively reviewed elsewhere [44-47]. For instance, Sekeris and colleagues were the first to discover the presence of GR in mitochondria [48]. Through its acute non-genomic effects, GCs promote mitochondrial apoptotic pathways resulting in the disruption of the mitochondrial membrane-potential and the release of pro-apoptotic factors such as Cytochrome C [49]. Importantly, the translocation of GR from the cytoplasm to the mitochondria correlates with the sensitivity of a given cell type to GC-induced apoptosis [50, 51]. In line with this, recent studies in mouse thymocytes showed that short term treatment with GC induces a direct interaction of GR with the pro-apoptotic Bcl2 family member associated proteins such as Bim [52]. Such interaction subsequently activates Bax decreasing thereby the mitochondrial membrane potential, Cytochrome C release, and Caspase-9 activation. However, it important to note that the effects of GC on the mitochondria control of apoptosis involve also genomic pathways. For example, in murine neuronal stem cells, dexamethasone was able to augment 2,3-methoxy-1,4-

naphthoquinone-induced apoptosis where a large percentage of studied genes involved in the mitochondrial respiratory chain and some encoding for anti-oxidant enzymes were downregulated by long-term treatment with GC [53]. These events allowed GCs to increase cellular sensitivity to oxidative stress promoting thereby neurotoxicity. This is clinically relevant as it can occur during prenatal exposure of the fetal brain to excess GCs [53].

Potential role of a putative mGR in mediating the rapid effects of GCs.

As previously described, the rapid non-genomic effects can, at least in part, be mediated through a putative mGR. Over the years, caveolin-1 (Cav-1), the major protein component of caveolae, has been implicated as a scaffold for the organization of several cytoplasmic signal complexes at the plasma membrane [54, 55]. In lung epithelial cells (A549), dexamethasone treatment leads to a rapid (within 2 min) phosphorylation of Cav-1 and protein kinase B (PKB)/Akt in a Src-dependent fashion [56]. Subcellular fractionation revealed co-localization of GR and Src to caveolin-containing membrane fractions [56]. Interfering with caveolae/caveolin (by disruption of lipid raft formation, impairment of function using dominant negative caveolin, down regulation of Cav-1 using shRNA, or genetic ablation of Cav-1) prevented acute (within 2 min) GC-induced PKB phosphorylation. Of note, caveolin down-regulation had little effect on GC-mediated transactivation, supporting the existence of a putative mGR. Further functional studies in caveolin knockout cells revealed considerable inhibition of GC-mediated cell growth arrest, suggesting that membrane-proximal signals acutely initiated by GC are required to mediate delayed effects (anti-proliferative effects) previously ascribed exclusively to the nuclear actions of GR [56]. Further evidence supporting a role for caveolae in mGR function stems from studies of membrane nuclear receptors such as estrogen receptor (ER) [57] showing requirement of Cav-1 in mediating acute cellular actions. Indeed, using epitope proximity ligation assays, Watson and colleagues demonstrated interactions of ERα with Cav-1. Interestingly, the use of nystatin, which binds to cholesterol and disrupts caveolar structures, blocked estrogen-induced rapid (5 min) ERK activation in pituitary tumor cells [57]. Together these findings indicate a critical role of Cav-1 in acute nuclear receptor/steroid signaling.

While the expression of mGR has been demonstrated in a myriad of cell types [58], the co-localization and cross-talk between mGR and Cav-1 is variable and highly cell-specific. Indeed, in U2-OS and MCF-7 cells, double recognition proximity ligation assays demonstrated the physical association of Cav-1 with the mGR [58]. However, studies in human $CD14^+$ monocytes

showed that mGR and Cav-1 are not co-localized and overexpression of the recombinant Cav-1 transcript in human K562 chronic myelogenous leukemia cells did not affect mGR expression/appearance suggesting that in these specific cell lines Cav-1 is not the limiting factor for mGR expression/appearance, without ruling out the possibility that it is a component of the transport machinery of GR from the cytosol to the membrane [59]. Palmitoylation, a critical post-translational modification occurring through the addition of fatty acid (e.g. palmitic acid) on amino acid residues of membrane proteins, plays a major role in the subcellular trafficking of proteins between membrane compartments [60]. Interestingly, the involvement of palmitoylation in the recruitment of other nuclear receptors, such as ER, to the plasma membrane has been reported [61]. Recent studies investigated whether this process is necessary for the recruitment of GR to the membrane and its co-localization with Cav-1 in COS-7 cells. Treatment of cells with the palmitoylation inhibitor, 2-bromopalmitate, had little effect on membrane localization of GR and its co-localization with Cav-1, and little influence on the acute effects of GC on MAPK signaling pathways. In addition, human GRα did not undergo S-palmitoylation, rendering this process unlikely to modulate membrane recruitment of GR [62]. Future studies on the mechanisms underlying GR recruitment to caveolae rich parts and its potential association with Cav-1 are warranted, specifically in airway cells.

Several studies have reported an interaction of mGR with other membrane receptors, particularly GPCRs [63]. Zhang and colleagues demonstrated the involvement of mGR and GPCR-dependent mechanisms in the rapid effect (as early as 1 min) of corticosterone on NMDA-evoked currents in hippocampal neurons [63] and further suggested that mGR may couple to multiple G proteins, including G_s and $G_{q/11}$. Other studies indicate that mGR directly elicits the activation of downstream intracellular signaling pathways. For instance, corticosterone might act via mGR to rapidly elicit PKC-dependent activation of ERK1/2 MAPK pathway (with 15 min) in PC12 cells [39]. Interestingly, proteomic analysis of the lymphoma cell line CCRF-CEM identified 128 proteins that were differentially regulated by the specific activation of mGR using BSA-conjugated cortisol for a short-term period (5 and 15 min) [58]. These actions were unique to mGR, as no activation of cGR target genes, such as GILZ, were observed. The majority of networks rapidly activated by mGR were mainly involved in cellular growth and cancer (after 5 min treatment with cortisol-BSA), cellular development, or hematological system development and function (after 15 min treatment with cortisol-BSA). Ingenuity pathway analysis provided strong evidence that mGR

is involved in pro-apoptotic, immune-modulatory, and metabolic pathways that are also regulated by GCs through cGR, suggesting that acute mGR stimulation can trigger rapid early priming events, ultimately paving the way for the slower genomic activities by GCs [58].

Concluding Remarks and Future Perspectives.

Although we have some insight in how GCs regulate different signaling pathways in a non-genomic fashion, future in depth investigations are warranted to further unravel details of these complex interactions. Indeed, key questions (see Outstanding Questions) still need careful consideration and additional research must address several important issues: i) the differential nature of non-genomic effects of GC in immune cells versus non-immune/structural cells; ii) differences between non-genomic effects of various steroids based on their lipophilicity [10]; iii) the fact that not all non-genomic effects are GR-mediated (RU486 insensitive) and may be due to non-specific interactions of GC with the cell membrane [2]; iv) the possibility that non-genomic and genomic effects are interconnected, where the acute non-genomic effects pave the way for the slower genomic activities of GCs [58]; and v) the significant role of Cav-1, and possibly other scaffolding/anchoring proteins, as a modulator of mGR activation, where the relative numbers of mGR associated with Cav-1 are critical in mediating non-genomic effects of GC [58, 64, 65]. Since **side effects** associated with GC therapy are often generated through its genomic actions [66], uncovering the non-genomic actions of GC with beneficial effects will likely lead to the development of compounds that selectively activate non-genomic signaling and thus have improved therapeutic profiles.

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Text boxes.

Box 1: Calcium regulation, ASM tone, and asthma pathogenesis.

Because airway smooth muscle (ASM) serves as the pivotal tissue regulating the bronchomotor tone, changes in the pathways regulating ASM contractile properties may play an important role in the development of abnormal lung function in asthma. Abnormal G-protein-coupled receptor (GPCR)-associated calcium homeostasis and ASM shortening may represent one of such mechanisms. Changes could occurs that at different levels of the contraction cascade including i) $[Ca^{2+}]i$ release from internal stores, ii) myosin light-chain kinase (MLCK) activity, iii) myosin light chain phosphorylation (pMLC), and iv) actin-myosin crossbridge cycling leading to cell shortening. Changes in ASM shortening could also be due to changes in sensitivity of the contractile apparatus to $[Ca^{2+}]i$ initiated by the small GTPase, RhoA, which activates Rho kinase (ROCK) to inactivate myosin light chain phosphatase (MLCP). Decreased MLCP activity results in an increase in pMLC levels for a given level of $[Ca^{2+}]i$, and thus enhancing ASM contractility. It is important to note that there are other parallel pathways where actin polymerization also mediates agonist-induced ASM shortening independently from Ca^{2+} and pMLC but potentially through the phosphorylation of other proteins such as vinculin. Collectively, this evidence suggests that ASM contractile function can mediate airway hyperresponsiveness in chronic airway inflammatory diseases by involving, at least partially, changes in Ca^{2+}-regulatory pathways.

Box 2. Role of NOS/NO signaling in asthma pathogenesis.

Altered NO production has been implicated in the development of both acute and chronic allergen-induced AHR. Production of NO occurs through the action of nitric oxide synthase (NOS), of which 3 isoforms have been identified thus far: two constitutive (c)NOS isoforms referred as neuronal (n), endothelial (e) NOS, and one inducible isoform called (i) NOS. Upon activation, cNOS isoforms produce relatively low amounts of NO, whereas iNOS can produce high and potentially damaging levels of NO. Whereas NO generated by eNOS is associated with beneficial bronchodilatory effects in allergic asthma, iNOS-derived NO is generally considered detrimental, as it has been linked to for instance epithelial damage, inflammatory cell infiltration, and mucus hypersecretion. These detrimental effects are largely due to the accumulation of Reactive Nitrogen

Species (RNS), including peroxynitrite, which are reaction products of NO and superoxide anions. Since NOS/NO signaling and RNS play key roles in chronic airway inflammatory diseases, including asthma and COPD, an acute role for GC/GR signaling and (inducible and/or endothelial) NOS activity can be envisioned. In depth studies are warranted to determine whether such functional interaction exists and whether or not targeting it would provide any therapeutic benefit for asthma.

546 **Tables.**

GC effects	Acute (simultaneous or within 30 min)	Chronic (delayed)
Genomic effects	-	+
Inhibitory effects of CHX or Actinomycin D	-	+
GR involvement	- or +	+
Inhibitory effects of RU486	- or +	+
Type of GR involved	None, membrane GR or cytosolic GR	Cytosolic GR
GR-independent mechanisms	GC interaction with membrane	None

547

548 **Table 1**: Various criteria (either alone or in combination) used to distinguish genomic effects from
549 non-genomic effects of glucocorticoids.

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551

Cell types	GCs	References
• Human bronchial epithelial cells	Dexamethasone Triamcinolone Hydrocorticone	3, 4, 10
• Rat thymocytes	Methylprednisolone	5
• Mouse neuroblastoma cells	Corticosterone	6, 14
• Cichlid fish pituitary cells	Cortisol	7
• Mouse cortical collecting duct cells	Dexamethasone Aldosterone	11
• Rat vascular smooth muscle cells	Aldosterone Cortisol Dexamethasone	12, 13, 25
• Rat B103 neuroblastoma cells	Hydrocorticosone	15
• Guinea-pig cochlear spiral ganglion neurons	Dexamethasone	16
• Rat hippocampal neurons	Corticosterone Dexamethasone BSA-conjugated cortisol	19
• Mouse skeletal C2C12 cells	Dexamethasone	20
• Guinea-pig tracheal tissues	Budesonide	22
• Murine airway smooth muscle cells	Dexamethasone	23
• Guinea-pig mouse model of allergic asthma	Budesonide	24
• Human vascular endothelial cells	Dexamethasone	30

552

553 **Table 2**: Examples of the various cells types where GCs were reported to have non-genomic
554 effects due their rapid onset, insensitivity to GR blockade (RU486), and protein synthesis
555 inhibition (cycloheximide).

Signaling pathways	Cell types	GCs	References
• PKA • SERCA Ca2+-ATPases • Adenylyl cyclase	Human bronchial epithelial cells	Dexamethasone	10
• PKC	Mouse cortical collecting duct cells	Dexamethasone Aldosterone	11
• IP3 accumulation • PKC	Rat vascular smooth muscle cells	Dexamethasone Aldosterone	12
• PKA	HT4 neuroblastoma cells	Corticosterone	6
• PKC	Rat B103 neuroblastoma cells	Corticosterone	15
• CaMKII • AMPK	Mouse skeletal myotubes	Dexamethasone	20
• PKC	Tracheal smooth muscle tissues	Cortisol	21
• Rho kinase	Rat vascular smooth muscle cells	Dexamethasone	25
• ROS/RNS (NO synthase)	Human breast cancer cells	Cortisol	26
• NO pathways	Guinea-pig cochlear spiral ganglion neurons Human vascular endothelial cells Human umbilical endothelial cells	Dexamethasone	16, 30, 33
• ERK1/2, P38MAPK, JNK	PC12 cells Rat vascular smooth muscle cells	Dexamethasone	25, 37

• Src tyrosine kinase	Human breast cancer cells A549 cells	Cortisol Dexamethasone	26, 40
• PI3K/Akt	Human vascular endothelial cells	Dexamethasone	30, 33

Table 3: Examples of signaling pathways activated by GCs via nongenomic mechanisms that were acute, sensitive (or insensitive) to GR blockade (RU486), and insensitive to protein synthesis inhibition (cycloheximide).

Figure legends.

Figure 1. Acute non-genomic effects of GCs on basal and agonist-induced Ca^{2+} responses. GCs have been described to differentially affect basal intracellular Ca^{2+} ($[Ca^{2+}]i$) homeostasis. Depending on the cell type studied and GC applied, GCs can either reduce or augment basal $[Ca^{2+}]i$. (A) GCs may decrease $[Ca^{2+}]i$ by activating AC/PKA mediated mechanisms, likely through events taking place at the cell membrane level and independent of GR stimulation, ultimately leading to SERCA activation (thapsigargin-sensitive Ca^{2+}-ATPase). (B) Conversely, GCs can activate PLC/IP3 and PKC dependent signaling cascades resulting in enhanced basal $[Ca^{2+}]i$; the involvement of GR in this process is currently unknown. (C) Agonist-induced increases in $[Ca^{2+}]i$ can be counteracted by GC-mediated activation of AC/PKA-induced stimulation of SERCA pumps as described in ATP stimulated cells. In contrast, a functional role for PKC was determined in the effects of GC on serotonin-induced Ca^{2+} responses, suggesting that the acute inhibitory mechanisms of GCs are highly agonist specific. (D) Limited studies are available on acute potentiating effects by GCs on agonist-induced Ca^{2+} responses; in neuronal cells it was suggested that these effects are mediated via the rapid activation of Ca^{2+}-influx through ionotropic ATP-gated purinergic 2X receptors. Whether glucocorticoid-mediated membrane receptors are involved in this pathway remains to be further investigated (mGR?). These responses rely on the presence of external Ca^{2+}. Abbreviations: AC, adenylyl cyclase; AR, agonist receptor; IP3, inositol 1, 4, 5-triphosphate; GC, glucocorticoid; mGR, membrane glucocorticoid receptor; PKA, protein kinase A; PKC, protein kinase C; SERCA, sarco/endoplasmatic reticulum Ca2+ -ATPase.

Glossary.

Muscle reactivity: The ability of the muscle to respond to contractile agonists. It is impaired during pathophysiological conditions such as asthma.

Calcium mobilization: Intracellular process triggered by external stimuli (e.g. contractile agonists) where calcium is released to be engaged in different cellular functions such as increased muscle reactivity and contraction. Calcium is usually acquired from extra-cellular sources (calcium influx) or intracellular stores (e.g. endoplasmic reticulum).

Genomic action: Action that modulates the expression of genes. It involves transcriptional processes where an activated transcriptional factor translocates to the nucleus and bind gene promoters to modulate their expression. Such processes require certain time and are delayed.

Glucocorticoid receptor (GR): A nuclear receptor, which acts as a receptor and a transcriptional factor. It is primary located in the cytosol. Glucocorticoids, through their lipophilicity, diffuse across the cell membrane to bind GR in the cytosol. Such binding promotes the translocation of GR to the nuclear where it binds gene promoters to modulate their expression. As described in this article, several evidence demonstrate a membrane version of GR, not acting as a transcriptional factor, but rather as a membrane receptor modulating the acute non-genomic effects of GC.

Non-genomic Action: Action that does not modulate the expression of genes. It does not involve transcriptional processes or protein synthesis. Such action promotes rapid effects on events proximal to the cell membrane to activate certain signal transduction pathways.

Side effects of GC: Due to their wide range of actions that include effects on the immune system, metabolism, skeletal muscle, bone and eyes, to name just few, GC exert in addition to its intended effect, some harmful effects especially when used in high dose and in long-term like in asthma patients. Such effects usually require the genomic actions of GR.