

Anabolic Androgenic Steroids and Intracellular Calcium Signaling: A Mini Review on Mechanisms and Physiological Implications

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Abstract: Increasing evidence suggests that nongenomic effects of testosterone and anabolic androgenic steroids (AAS) operate concertedly with genomic effects. Classically, these responses have been viewed as separate and independent processes, primarily because nongenomic responses are faster and appear to be mediated by membrane androgen receptors, whereas long-term genomic effects are mediated through cytosolic androgen receptors regulating transcriptional activity. Numerous studies have demonstrated increases in intracellular Ca^{2+} in response to AAS. These Ca^{2+} mediated responses have been seen in a diversity of cell types, including osteoblasts, platelets, skeletal muscle cells, cardiac myocytes and neurons. The versatility of Ca^{2+} as a second messenger provides these responses with a vast number of pathophysiological implications. In cardiac cells, testosterone elicits voltage-dependent Ca^{2+} oscillations and IP_3R -mediated Ca^{2+} release from internal stores, leading to activation of MAPK and mTOR signaling that promotes cardiac hypertrophy. In neurons, depending upon concentration, testosterone can provoke either physiological Ca^{2+} oscillations, essential for synaptic plasticity, or sustained, pathological Ca^{2+} transients that lead to neuronal apoptosis. We propose therefore, that Ca^{2+} acts as an important point of crosstalk between nongenomic and genomic AAS signaling, representing a central regulator that bridges these previously thought to be divergent responses.

Keywords: Androgens, Ca^{2+} , cardiac myocytes, heart, genomic, neurons, nongenomic, skeletal muscle cells, testosterone.

1. INTRODUCTION

Second messengers are ions or molecules responsible for the transduction of extracellular signals into cellular processes. Among these, the Ca^{2+} ion is the most ubiquitous second messenger signal in eukaryotic systems and represents one of the most complex messages decoded by cells. Over the past decades, the study of intracellular Ca^{2+} has evolved from the observational study of Ca^{2+} transients inside different types of cells to becoming the basis of a vast array of physiological and pathological processes. For instance, we currently know that cardiac function depends almost entirely on fine-tuning of intracellular Ca^{2+} , which when imbalanced leads to arrhythmias, cardiac failure or stroke. In neurons, Ca^{2+} regulates many processes including gene expression or the synaptic release of vesicles. In these, and many other types of cells, the regulation of intracellular Ca^{2+} is key for the determination of life-death outcomes,

which contribute to neuropathies, cell death or tumorigenesis. Therefore, understanding the physiology of Ca^{2+} -regulated processes is essential for finding new approaches to the treatment of human diseases including those of highest mortality in developed countries: cardiovascular disease, cancer and neurodegeneration, as well as many other genetic or endocrine disorders.

Steroidal hormones such as testosterone and its derivatives are well known for their androgenic properties and anabolic effects. The cumulative effect of these hormones is to direct the differentiation of organs and tissues toward the adoption of male phenotypes. Classically, anabolic androgenic steroids (AAS) act through binding to androgen receptors (AR), which once bound by their ligands, function as nuclear transcription factors promoting the expression of genes under the control of steroid-response elements (SRE). This programmed gene expression is achieved within a time course of hours after AAS binding to ARs. More recently, however, it has been described that steroidal hormones including AAS can also provoke faster responses, which do not involve gene expression. These effects have been termed as 'nongenomic', and they cover a wide range of intracellular processes such as the activation of

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membrane bound receptors, triggering of downstream pathways that involve protein kinases and phosphatases, mobilization of intracellular Ca²⁺, as well SRE-independent changes in transcription [1]. The origin of these responses has been attributed to AR-AAS complexes present in caveolin-enriched zones of the plasma membrane [2], however, recent studies identify orphan candidates for membrane-bound AR that after binding to AAS, trigger activation of intracellular second messengers [3]. This fascinating emerging topic will certainly contribute to our understanding of the pathophysiological consequences of androgen action. In the next sections, we will focus our attention in those nongenomic actions of AAS that are related to Ca²⁺ signaling, with emphasis on such effects on cardiac muscle cells, skeletal muscle cells and neurons. We will discuss the mechanistic pathways involved, their pathological implications and their perspectives.

2. AAS AND CALCIUM

2.1. Brief Description of Cellular Ca²⁺ Toolkits

Cellular Ca²⁺ signals possess the unique features of spatial compartmentalization and temporality, which together determines the message they carry and therefore the response they evoke. For instance, very fast (milliseconds) and localized Ca²⁺ increases allow the fusion of presynaptic vesicles with the plasma membrane, leading to neurotransmitter release or receptor externalization. During muscle contraction, a series of complex, compartmentalized and sequential Ca²⁺ events provide a mechanism for the decoding of a depolarizing signal into the contraction of sarcomere fibers within fractions of seconds, a process known as excitation-contraction (EC) coupling. On another hand, Ca²⁺ waves propagated across the cytoplasm of many different cell types lead to the activation of Ca²⁺-sensitive effectors, which include Ca²⁺-binding proteins, Ca²⁺-sensitive kinases and Ca²⁺-sensitive transcription factors. Once initiated, these processes can operate in a temporal range that covers milliseconds, minutes and even hours in the case of gene expression. Such localized Ca²⁺ signals are tightly controlled by a highly specialized molecular toolkit, capable of increasing Ca²⁺ concentrations in specific zones of the cytosol or the nucleus, and of removing it very rapidly (for details please see [4]).

Briefly, Ca²⁺ can be either introduced to the cytosol from the extracellular medium, or released from internal Ca²⁺ stores such as the sarco-endoplasmic reticulum. Several different Ca²⁺ channels reside in the plasma membrane, and in response to a variety of stimuli, they increase their open probability state, allowing the influx of Ca²⁺ ions. Among these channels, the family of voltage dependent L-type Ca²⁺ channels is of interest here, because they are expressed in neurons, myotubes and cardiomyocytes, and constitute the first element to respond to a depolarizing signal. Upon opening, they produce localized cytoplasmic Ca²⁺ *sparklets*, which can be sensed by another Ca²⁺ channel resident in the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR), known as the ryanodine receptor (RyR). In the case of neurons, type 1 RyRs respond by releasing Ca²⁺ from the ER in the form of *syntilla*, which allows the fusion of presynaptic vesicles with the plasma membrane and the

release of neurotransmitters or hormones. In the case of cardiomyocytes, the opening of type 2 RyRs after a *sparklet* leads to SR Ca²⁺ release in the form of *sparks*, which stimulate the contraction of sarcomeres resulting in muscle contraction. In skeletal muscle and cultured myotubes, there is a direct interaction between L-type Cav1.1 channels and type 1 RyR facilitating the triggering of Ca²⁺ release.

Another ER-resident Ca²⁺ channel is the inositol 1,4,5-trisphosphate receptor (IP₃R). Unlike RyRs, these channels do not crosstalk with L-type Ca²⁺ channels. Instead, they are ligand-activated in response to inositol 1,4,5-trisphosphate (IP₃), which is produced in the inner face of the plasma membrane by the enzyme phospholipase C (PLC). Several isoforms of PLC exist, according to the type of plasma membrane receptor to which they are coupled. Once IP₃ is bound to IP₃Rs, these channels respond by releasing Ca²⁺ from the ER in the form of *puffs* into the cytosol and/or into the nucleus. These localized events lead to the activation of several effectors, including Ca²⁺-binding proteins such as calmodulin, enzymes such as calcineurin and calmodulin kinase, as well as Ca²⁺-sensitive transcription factors such as NFAT or CREB [5, 6]. Ca²⁺ can also be propagated throughout the cytoplasm in the form of *waves* through a crosstalk between aligned IP₃Rs, in which Ca²⁺ itself increases the opening probability of surrounding IP₃Rs.

The mechanisms to extrude Ca²⁺ from the cytosol include ATP-dependent pumps, such as the plasma membrane and the sarco-endoplasmic reticulum Ca²⁺ ATPases (PMCA and SERCA respectively), as well as the plasma membrane Na⁺/Ca²⁺ exchanger (NCX). Mitochondria represent an additional Ca²⁺ buffering organelle, as a substantial load of Ca²⁺ released from the ER is driven into the mitochondrial matrix by the voltage-dependent anion channel (VDAC) and the mitochondrial Ca²⁺ uniporter (MCU). In this way, Ca²⁺ is removed rapidly from the cytosol, thus shaping an undetermined set of Ca²⁺ signals that encode a vast array of messages (Fig. 1). For instance, slow Ca²⁺ signals in the cytoplasm and nuclei after electrical stimulation of skeletal myotubes depend on IP₃ receptors [7-9]. This slow Ca²⁺ signal is also modulated by mitochondria [10], and activates a specific program of gene expression. Equivalent signals have been observed in adult skeletal muscle fibers [11].

2.2. Acute Effects of AAS on Intracellular Ca²⁺

Before focusing on the relationship between steroids and intracellular Ca²⁺, one particular aspect of this relationship is the direct effect of AAS on Ca²⁺ channels. Increasing evidence indicates that L-type calcium channels are regulated by AAS in two different ways: acutely, by a direct antagonistic effect of AAS upon L-type Ca²⁺ channels, and chronically, after a genomic response that leads to increased expression of L-type Ca²⁺ channels [12, 13]. Relative to the structure-activity relationship, the Peers' group seems to have done the most research on this subject, using HEK 293Ar75 cells [14-16]. In a series of patch clamp experiments, among the different steroids tested, only testosterone blocked L-type calcium channels. There are three interesting observations to highlight these findings, the first one being that despite the structural similarity among all steroids assayed, only testosterone blocked the channel;

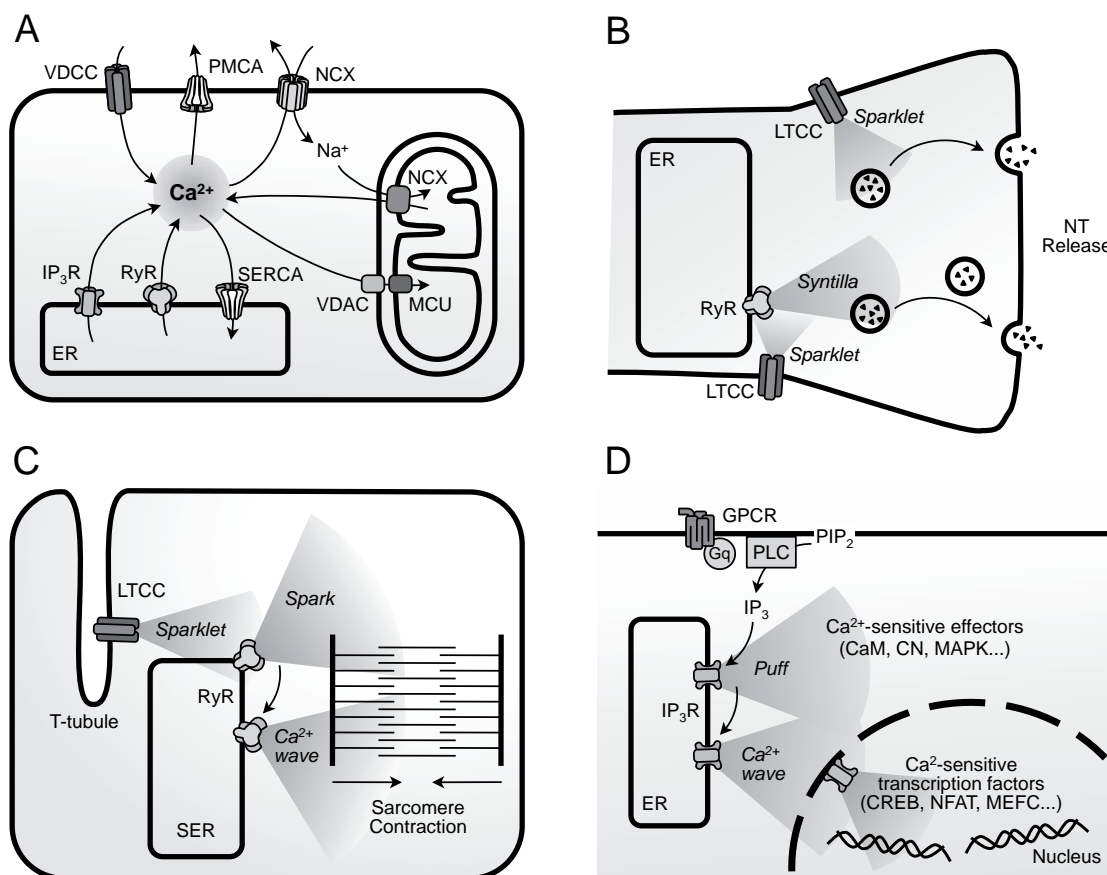


Fig. (1). Intracellular Ca^{2+} signaling at a glance. **(A)** General vision of the basic Ca^{2+} toolkit. Mechanisms that drive Ca^{2+} into the cytosol from the extracellular medium include ligand-gated Ca^{2+} channels (not shown) and voltage-dependent Ca^{2+} channels (VDCC). Ca^{2+} -release mechanisms from intracellular stores include inositol 1,4,5-trisphosphate receptors (IP_3R), ryanodine receptors (RyR) and the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). Mechanisms to extrude Ca^{2+} from the cytosol include the plasma membrane NCX, the plasma membrane and the sarco-endoplasmic reticulum Ca^{2+} -ATPases (PMCA and SERCA, respectively), as well as the mitochondrial outer membrane voltage dependent anion channel (VDAC) and the mitochondrial inner membrane Ca^{2+} uniporter (MCU). **(B)** Ca^{2+} microdomains regulate neurotransmitter release in neurons. L-Type Ca^{2+} channels (LTCC) introduce Ca^{2+} into the cytosol in the form of *sparklets*, which directly induce local vesicle fusion with the terminal, or activate RyRs to open and release higher amounts of Ca^{2+} from the endoplasmic reticulum (ER) in the form of *syntilla*, which elicits massive neurotransmitter release. **(C)** Ca^{2+} microdomains regulate sarcomere contraction in skeletal and cardiac muscle cells. Depolarizing signals in T tubules induce opening of LTCCs. The resulting *sparklet* stimulates the opening of RyRs in these cells, which release Ca^{2+} in the form of *sparks*. This Ca^{2+} increase propagates as Ca^{2+} waves to generate muscle contraction. **(D)** The general mechanism for IP_3R -mediated Ca^{2+} release in response to G protein-coupled protein receptors (GPCR) involves activation of phospholipase C (PLC) and local production of IP_3 , which activates IP_3Rs to release high amounts of Ca^{2+} from the ER in the form of *puffs*. These signals lead to activation of Ca^{2+} -dependent effectors in the cytosol, including calmodulin (CaM), calcineurin (CN) and mitogen-activated protein kinases (MAPK), among others. The propagation of *puffs* also leads to Ca^{2+} waves, which encode different messages according to their amplitude and frequency. Nuclear IP_3R -mediated Ca^{2+} *puffs* or *waves*, lead to the activation of Ca^{2+} -dependent transcription factors, as illustrated.

second, considering the structural requirements, none of these structures resembles those of established Ca^{2+} channel blockers such as dihydropyridines, phenylalkylamines or benzothiazepines; and third, the researchers suggest that testosterone and nifedipine share common molecular requirements for inhibition of L-type Ca^{2+} channels in the S5 region of domain III [15]. It is important to note that the binding sites described for dihydropyridines are in segments 5 and 6 of domain III, and 6 of domain IV [17]. However, no specific binding region has been identified for AASs so far. Irrespective of these details, the acute effects of androgens upon L-type Ca^{2+} channels provide a potentially useful

clinical strategy for the treatment of myocardial ischemia [18], coronary artery disease [19, 20], as well as for the reduction of cholesterol and visceral adiposity [21-23]. These effects resemble those of third generation dihydropyridines such as amlodipine; further structural studies will contribute to the elucidation of the direct mechanisms involved in these responses. Thus far, the effects of androgens on the cardiovascular system remain incompletely understood, and some studies point to increased cardiovascular risk associated with high circulating androgen levels [24, 25], which is related to genomic responses that lead to cardiomyocyte hypertrophy [26], high

L-type Ca²⁺ channel expression [12] and blood pressure [27, 28]. That said, other studies support a long-term cardioprotective role [29, 30]. Despite these differences, increasing clinical evidence supports consideration of transient use of testosterone derivatives to improve cardiovascular and pulmonary status [31].

2.3. Calcium-Mediated Myocyte Hypertrophy

The role of Ca²⁺ in mediating hypertrophy of cardiomyocytes and skeletal muscle cells has been well documented [32, 33]. Intracellular Ca²⁺ elevations trigger myocyte hypertrophic signaling *via* the phosphatase calcineurin, activation of the transcription factor NFAT [34] and the cyclic T-cdk9 complex [35]. Other transcription factors such as CAMTA2 are essential for regulating cardiac hypertrophy in response to calmodulin signaling, *via* the association with class II histone deacetylases (HDACs) [36]. Among the other Ca²⁺-dependent transcription factors involved in myocyte hypertrophy is CREB [37] and MEF2C [38, 39]. Interestingly, the AR has also been shown to induce myocyte hypertrophy [40] and independently, to regulate the activity of MEF2C [41], which is also regulated by calcineurin [42]. Therefore, a reasonable and predicted link between these processes could be mediated by androgen-induced Ca²⁺ signaling. Interestingly, Ca²⁺ triggers cardiac hypertrophy in response to several other agents including angiotensin II, endothelin-1, α -adrenergic agents and mechanical stretching [43]. However, it is currently unknown how androgens regulate signal transduction cascades during cardiac hypertrophy, and Ca²⁺ is a prominent mechanism that could drive these effects in the heart and in skeletal muscle cells.

Nongenomic testosterone effects have been observed in skeletal muscle cells, *via* the release of Ca²⁺ from internal stores [44, 45]. This Ca²⁺-wave signal involved a G protein-coupled membrane receptor and the activation of PLC with IP₃ production and IP₃R-mediated Ca²⁺ release from internal stores. Ca²⁺ release induced by testosterone also activates a Ras/MEK/ERK pathway, which is known to be involved in the extracellular response to growth factors that elicit hypertrophic processes in myocytes [46]. In cardiac myocytes, these effects were mimicked by cell-impermeant testosterone complexed to albumin (T-BSA), and were not inhibited by the intracellular AR antagonist cyproterone. This result suggests that a membrane-resident AR different from the classic AR mediates these responses. Interestingly, this Ca²⁺ signal was complex and demonstrated to contain at least 3 components [47]. First, in the presence of extracellular Ca²⁺, cardiac myocytes stimulated with testosterone enhanced their basal Ca²⁺ levels *via* a mechanism that involves L-type Ca²⁺ channels. Second, the magnitude of the basal Ca²⁺ oscillations was enhanced by testosterone, in a pathway that involves crosstalk between L-type Ca²⁺ channels and RyRs. Third, in the absence of extracellular Ca²⁺, cardiac myocytes evidenced a G $\beta\gamma$ /PLC/IP₃/IP₃R pathway that leads to a nuclear Ca²⁺ with kinetics faster than the cytosolic Ca²⁺ increases.

From these responses, multiple perspectives emerge. On one hand, the cardiomyocyte contraction process mediated by Ca²⁺-induced Ca²⁺ release could be inotropically

enhanced, as the basal oscillations were increased in magnitude but not in frequency. On the other hand, the increase in nuclear Ca²⁺ is likely to be involved in the modulation of gene expression. Further elucidation of these processes will certainly reveal new pathways in the hypertrophic response of cardiac myocytes to AAS. However, new insights have already been obtained regarding the relationship between cytosolic Ca²⁺ levels and the activation of the mammalian target of rapamycin (mTOR) complex 1, which is a master regulator of cell growth by transcriptional activity and protein synthesis [48]. In cultured cardiac myocytes, both the MEK/ERK pathway and the mTOR/S6K axis are stimulated by testosterone, leading to increased cell size, amino acid incorporation, and augmented expression of β -myosin heavy chain and α skeletal actin in an IP₃R/Ca²⁺-dependent fashion [26]. These results lend yet more evidence to a model where Ca²⁺ serves as a novel central regulator of cardiac myocyte hypertrophy in response to AAS. Thus, nongenomic responses mediated by Ca²⁺ operate in concert with transcriptional responses, establishing a functional link between these processes which were previously thought to be independent (Fig. 2). In concordance with these findings, the non-permeant T-BSA complex has been shown to promote actin cytoskeleton reorganization *via* nongenomic responses in prostate cancer cells [49]. Additionally, Ca²⁺ transients have been reported to increase the binding of testosterone to intracellular ARs [50]. Further studies will elucidate more details of the triangular interplay among androgens, Ca²⁺ and hypertrophy, in particular regarding the participation of classical hypertrophy-related transcription factors. With time, we expect these insights to be relevant to skeletal muscle cells as well.

2.4. Calcium Oscillations and Neuronal Apoptosis

The brain represents a hallmark target site for the action of steroidal hormones. Both adrenal and gonadal steroids play important roles in brain development and function *via* genomic and nongenomic mechanisms [1, 51-55]. In addition, neurons constitute an elegant and complex model for the study of Ca²⁺ signals, mainly because Ca²⁺ regulates essential neural processes including synaptic plasticity [56], exocytosis [57], gene expression [58, 59], bioenergetics [60], autophagy [61] and apoptosis [62]. It has been described that estrogen promotes survival of neurons [63]; and mitochondrial Ca²⁺ levels seem to participate in these responses *via* metabolic mechanisms [64]. In contrast, testosterone may exert opposite effects upon neuronal survival, for instance by decreasing neural function and the mass of dopaminergic neurons in animal models [65]. These findings are consistent with the neuronal loss observed on human AAS abusers [66], as well as with the augmented risk of developing psychiatric disorders among these individuals [67]. On the other hand, it has been described that high doses of AAS induce apoptotic cell death in cardiac myocytes [68]. Interestingly, testosterone-driven changes in intracellular Ca²⁺ levels are similar in cardiac myocytes and neurons, namely rapid increase in Ca²⁺ oscillations.

Ca²⁺ oscillations represent, in comparison to single Ca²⁺ transients, a more complex signal to decode by cells, basically because they contain additional information

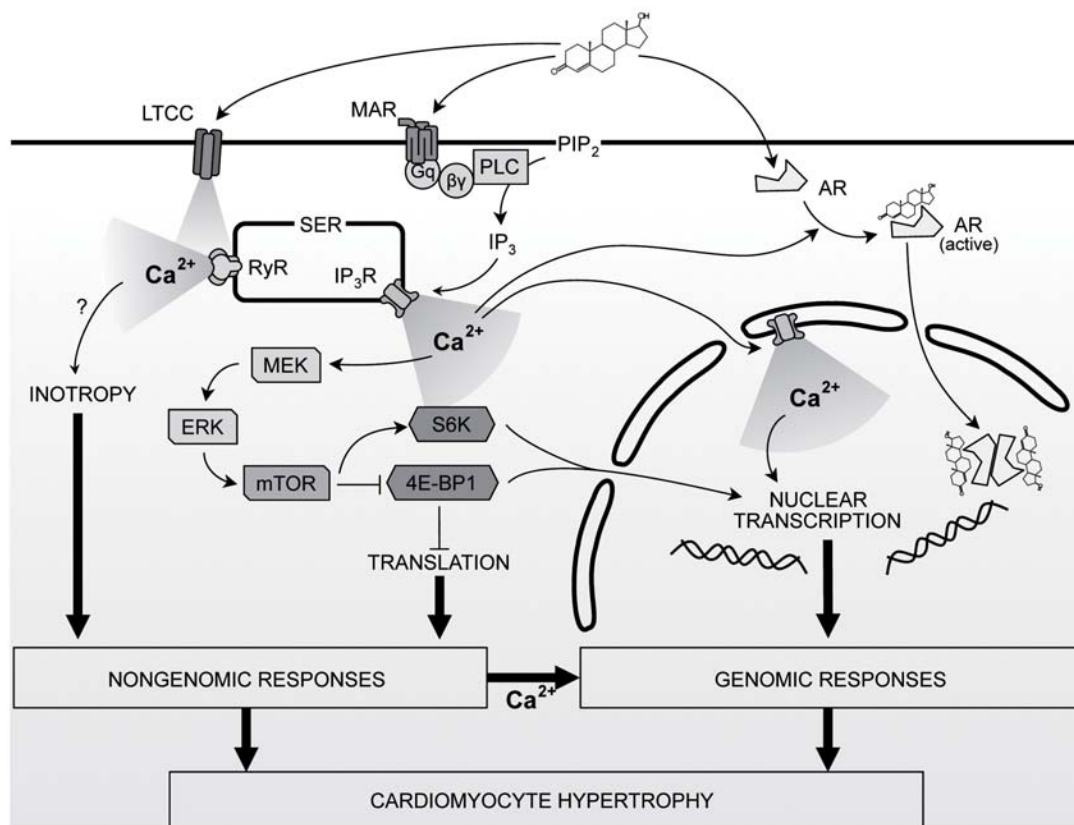


Fig. (2). Effects of AAS on intracellular calcium. In myotubes, as well as in cardiomyocytes, testosterone activates plasma membrane androgen receptors (MAR) coupled to heterotrimeric Gq protein. G_{βγ} dimers activate phospholipase C (PLC), which locally produces inositol 1,4,5-trisphosphate (IP₃) thus activating IP₃ receptors (IP₃R). The consequent Ca²⁺ increase leads to the activation of several pathways, including the MEK/ERK/mTOR axis, propagation of nuclear Ca²⁺ signals and modulation of intracellular AR affinity for testosterone (an effect observed in platelets). These responses combined induce a series of genomic changes that lead to increased gene expression and increased protein synthesis, ultimately leading to cardiac hypertrophy. Although not studied in further detail, the effect at the left side of the figure upon Ca²⁺ release from ryanodine receptors (RyR), might lead to inotropy and therefore contribute *via* nongenomic changes to the hypertrophic phenotype. An overview of these processes is presented in the lower part of the figure, which illustrates how Ca²⁺ is centrally regulating the crosstalk between nongenomic and genomic responses.

encoded in their frequency and amplitude (for more details, please read [69]). These two parameters have been reported to activate transcriptional genomic responses [70, 71], mediate cardiac hypertrophy [72], or to enhance dendritic growth in neurons [73]. Transient testosterone addition to human neuroblastoma cells produces rapid intracellular Ca²⁺ oscillations that correlated with an increase in neurite growth. This response is dependent on concerted interactions between Ca²⁺ release through type-1 IP₃R and Ca²⁺ influx from the extracellular medium [74]. Importantly, these responses were obtained with 100 nM testosterone, a concentration that is on the high circulating range observed in humans, and therefore depends on physiological conditions such as gender, the physical state, age, or brain aromatase levels. This increase in neurite growth might be related to brain development and brain plasticity events observed with advanced age. However, higher testosterone levels are usually found among individuals exposed to testosterone replacement therapy or AAS abuse. In concordance with this notion, it has been observed that concentrations of 1-10 μM testosterone lead to higher and sustained Ca²⁺ signals that produce neuronal apoptosis [75].

This neuronal cell death cascade requires caspase activation and is inhibited by pharmacological inhibition or siRNA-mediated depletion of type-1 IP₃R. Therefore, a dual response is produced by testosterone on neuronal cells, on one hand promoting synaptic plasticity at physiological concentrations, and on the other hand, leading to apoptosis at higher concentrations (Fig. 3). Further details of these mechanisms are urgently needed to understand these effects in terms of the genetic responses involved, the possible adaptive mechanisms to chronic androgen administration and the clinical consequences in individuals exposed to high levels of androgens.

2.5. Circadian Regulation of AAS Signaling

There is a growing appreciation for the importance of circadian rhythms in human health and disease. These 24-hour cycles anticipate changes in physiological demand and help to coordinate appropriate responses or provide temporal separation of opposing but equally important processes. In the case of AAS, there is evidence of circadian control at many levels ranging from timing of hormone release and receptor availability, to circadian changes in intercellular

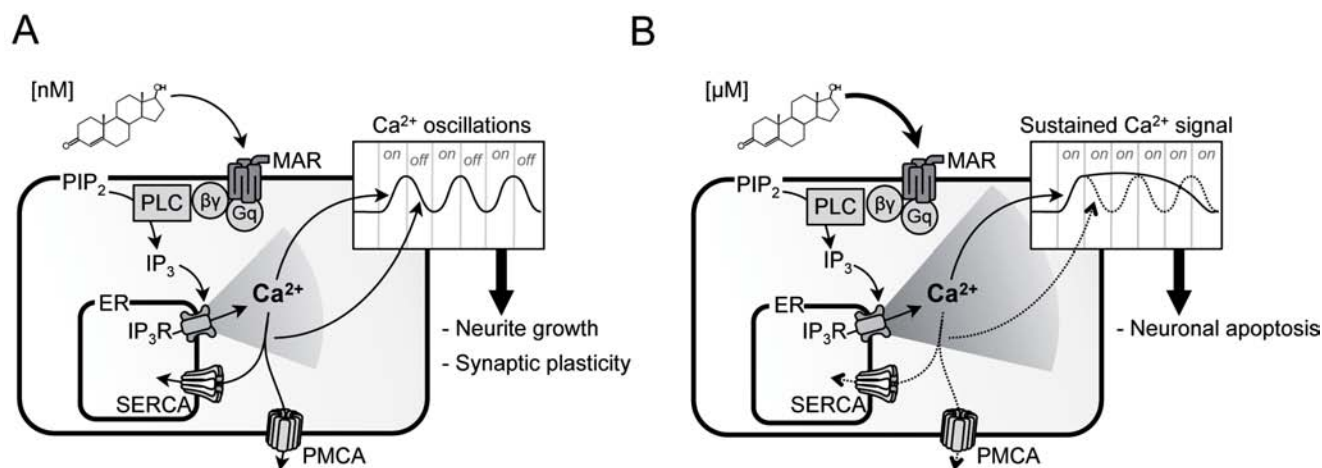


Fig. (3). Implications of Ca²⁺ oscillations in a neuronal model of AAS abuse. The study on the effects of testosterone upon neuroblastoma cells has provided two models for androgen action. **(A)** At physiological concentrations (nM), testosterone elicits intracellular Ca²⁺ oscillations, which are turned 'on' by inositol 1,4,5-trisphosphate receptor (IP₃R)-mediated Ca²⁺ release, and are turned 'off' by Ca²⁺ extruding mechanisms. These oscillations lead to an increase in neurite overgrowth, which might be related to synaptic plasticity. **(B)** At pathological concentrations (µM), testosterone persistence leads to enhanced activation of membrane androgen receptors (MAR), which lead to a sustained turning 'on' of the Ca²⁺ signal. Under these conditions, mitochondrial-dependent cascades of caspase activation lead to neuronal apoptosis.

Ca²⁺ levels. Conversely, testosterone can influence circadian day length and accordingly, sustained use of AAS has been associated with hyperactivity and disruption of circadian rhythm [76]. It is likely that this cross-regulation between AAS and circadian rhythms involves both genomic and nongenomic mechanisms of action, however, for the sake of this review we will touch briefly on the potential involvement of Ca²⁺ signaling. Cytoplasmic Ca²⁺ levels are circadian in a number of tissues including the suprachiasmatic nucleus (SCN), the region of the brain where the central clock mechanism resides, and Ca²⁺ acts as an important second messenger during clock resetting [77]. Immerging studies suggest that normally in the healthy heart, diastolic cytoplasmic Ca²⁺ levels are also circadian, which results in circadian activation of calcineurin and NFAT [78]. Cardiac myocytes require repair and maintenance, and this is most likely occurring during times of lower demand [79]. In mice, the peak observed in cardiac calcineurin/NFAT activity occurs at the beginning of the animal's resting phase and activation of this pathway is an important component of cardiac remodeling. Consistent with concept of cardiac rhythms in Ca²⁺ signaling, isolated adult cardiomyocytes display circadian variation in the magnitude of Ca²⁺ release in response to isoproterenol [80]. Similarly, both brain [81] and cardiac [82] tissue display diurnal rhythms in susceptibility to ischemic damage. As circulating levels of AAS are circadian, it is essential to consider their potential involvement in coordination of anabolic, catabolic, and cell survival cycles in order to fully understand their mechanism of action *in vivo*.

3. SUMMARY AND CONCLUDING REMARKS

Genomic responses of androgens, acting *via* binding to intracellular AR and changes in transcription, take hours to manifest, whereas a new set of rapid responses, termed 'nongenomic,' has been widely reported. Among the rapid

effects of androgens, changes in intracellular Ca²⁺ represent a particularly powerful signaling mechanism, because Ca²⁺ is a central mediator of a diverse array of cell responses including entrainment and maintenance of circadian rhythmicity. In both skeletal muscle cells and cardiac muscle cells, testosterone elicits a rapid release of Ca²⁺ from IP₃-dependent and IP₃-independent stores, which are related to ERK signaling, mTOR signaling, gene expression and possible inotropic effects [26, 44, 47]. Together, these responses lead to myocyte hypertrophy, thus bridging nongenomic and genomic responses *via* established Ca²⁺-dependent mechanisms. On the other hand, neurons are also a target for the action of steroidal hormones, and testosterone regulates several processes depending on its concentration. At physiological levels (nM), testosterone produces Ca²⁺ oscillations mediated by IP₃R and Ca²⁺ influx. These oscillations are responsible for enhancing neurite growth and thus might regulate synaptic plasticity during development or brain damage events. At pharmacological concentrations (µM), such as those found in individuals with androgen replacement therapy or AAS abuse, testosterone leads to prolonged and sustained Ca²⁺ transients, which unleash mitochondrial dependent caspase activation mechanisms and lead to neuronal apoptosis [74, 75]. With respect to the adverse cardiovascular consequences of chronic AAS treatment, these studies also provide new insights into the mechanisms responsible for increased blood pressure, increased risk of pathological cardiac hypertrophy, arrhythmias and stroke [24, 83]. Furthermore, due to the diversity of levels at which circadian-regulated processes can influence and cross-talk with AAS signaling, we propose that it will be essential to integrate circadian influences with genomic and nongenomic effects to fully understand the impact of AAS *in vivo*.

Thus far, intracellular Ca²⁺ signaling represents a significant and potentially new important effect of AAS in muscle cells, neurons and possibly many other cell types. Additional insights into the implication of these effects upon gene expression and long-term genomic responses are urgently needed, and are likely to reveal unexpected physiologically relevant links between nongenomic and genomic responses to androgenic steroids.

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ABBREVIATIONS

AAS	= anabolic androgenic steroids
AR	= androgen receptor
Ca ²⁺	= calcium
[Ca ²⁺]	= Ca ²⁺ concentration
E-C	= excitation-contraction
ER	= endoplasmic reticulum
ERK	= extracellular-regulated kinase
HDAC	= histone deacetylase complex
IP ₃	= inositol-1,4,5-trisphosphate
IP ₃ R	= IP ₃ receptor
LTCC	= L-type Ca ²⁺ channel
MAPK	= mitogen-activated protein kinases
MAR	= membrane AR
MCU	= mitochondrial Ca ²⁺ uniporter
mTOR	= mammalian target of rapamycin
NCX	= Na ⁺ /Ca ²⁺ exchanger
PIP ₂	= phosphatidylinositol 4,5-bisphosphate
PLC	= phospholipase C
PMCA	= plasma membrane Ca ²⁺ ATPase
RyR	= ryanodine receptor
SERCA	= sarco-endoplasmic reticulum Ca ²⁺ ATPase
SR	= sarcoplasmic reticulum
T-BSA	= testosterone covalently bound to albumin
VDAC	= voltage-dependent anionic channel
VDCC	= voltage-dependent Ca ²⁺ channel

SCN	= suprachiasmatic nucleus
S6K	= ribosomal protein S6 p70 kinase
4E-BP1	= translation initiation factor 4E binding protein 1

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