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NONGENOMIC ACTION OF STEROID HORMONES IN VERTEBRATES

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Steroid hormones regulate diverse biological functions by binding to intracellular receptors, which in turn alter the expression of genes. According to the common theory of steroid action, steroids modulate gene transcription by interacting with intracellular receptors, which act as ligand dependent transcription factors. Steroids regulate various genes either by positive or negative expression (1,2). Most of the steroid receptors are located in side the cell and hence the steroids need to get into the cells and alter gene expression. The receptors for glucocorticoid, mineralocorticoid, progesterone and androgen are cytoplasmic and that of estrogen is nuclear (3). These receptors exist in inactive state in the cytoplasm or nucleus. The inactive state is maintained by their interaction with a group of receptor associated proteins called the chaperones and cochaperones (4,5). The mode of action of steroids requires intracellular localization of the steroid receptor and typically takes at least 30 to 60 min for the response. These cellular responses of steroids are known as the classical genomic action of steroids, which are characterized by a specific delay and sensitivity towards inhibitors of transcription and translation. The detailed description of steroid actions was the result of intensive long term research on steroid hormones.

Recently, it has become evident that some of the steroid hormones' actions are not due to these classical genomic mechanisms since they occur too rapidly following steroid exposure and appear insensitive to the action of protein synthesis inhibitors. A rapid response to steroids that occurs within seconds or minutes and likely not to be mediated by the genome is termed nongenomic. The first description on nongenomic action of steroid was reported 60 years ago as the anesthetic effects of progesterone, which occurred almost immediately after the application of the hormone (6). In 1963, Klein and Henk (7) demonstrated that administration of aldosterone in men increased peripheral vascular resistance and blood pressure within 5 minutes, suggesting a nongenomic mechanism of action because of the short time frame. Almost concurrently, Spach and Streeten, in 1964 (8) reported *in vitro* effects of aldosterone on Na^+ exchange in dog erythrocytes. Lacking nucleus, the *in vitro* effects in these cells must be nongenomic in nature. Further more, rapid effect of glucocorticoids on isolated synaptosomes was recognized in the mid 1970s' (9). Advancement in this field has, however, been very slow until few years ago, when evidence at the cellular level started accumulating to support and further clarify this novel mode of action of steroids.

Nongenomic steroid actions have been studied in many eukaryotes including plants (10), and in diverse species of animals from crayfish to human (11-19). These steroid actions have been characterized as rapid effects lasting from milliseconds to less than an hour (20). Rapid onset, equally fast recovery after steroid removal, insensitivity to inhibitors of protein

synthesis, interaction at plasma membrane or membrane binding and transduction via rapid cellular mechanisms known to mediate peptide and neurotransmitter actions (14) are other important characters of nongenomic action. Early observations of rapid steroid actions have been expanded during recent years. Through advances over the past ten years, it is now clear that all classes of steroids including the secosteroid vitamin D_3 and thyroid hormones can rapidly alter physiological processes through nongenomic membrane associated mechanisms typically ascribed to the fast effects of neurotransmitters and peptide hormones (21-24).

Existence of nongenomic action of glucocorticoid (GC) is well established in vertebrates, especially, in homeotherms. Dallman and Yates, in 1969(25) observed a rapid feed back action of GC on pituitary, which occurred within 5 minutes after an increase in plasma level of GC. Glucocorticoid rapidly inhibited ascorbic acid uptake in sectioned pituitary cells (26). Electrophysiological and behavioural studies suggest that glucocorticoids might also rapidly modulate neuronal activity in the nervous system. In guinea pig, cortisol hyperpolarized the membrane potential and inhibited electrical discharges of isolated celiac ganglions within two minutes (27). In contrast, corticosterone enhanced neuronal firing rates of cardiovascular neurons in rat (28). Dexamethasone reversibly inhibited acetylcholine induced current amplitudes within several seconds in isolated guinea pig chromaffin cells (29). Several studies also support nongenomic action of glucocorticoids in the cerebral cortex of mammalian brain. Corticosterone stimulated depolarization dependent Ca^{2+} uptake within 15 seconds in brain synaptosomes and promoted calmodulin binding in non-nucleated synaptic membranes (30,31). The importance of nongenomic steroid actions to the whole organism is best demonstrated by the rapid behavioural responses seen during acute steroid exposure. In white crowned sparrows, non-invasive corticosterone treatment leads to rapid increases in behavioural activity (such as perch hopping) analogous to those responses seen during natural disturbances (32). Corticosterone rapidly suppressed male courtship clasping behavior in rough skinned newt *Taricha granulosa* (33). Administration of corticosterone, at concentration that mimics plasma levels, produced stress-increased locomotion within 15 minutes in rodents (34).

It is well known that glucocorticoids elicit a set of reflex responses necessary for maintaining homeostasis, particularly during periods of stress. In the absence of a negative feed back, unchecked glucocorticoid activity can prove hazardous, leading to a collapse of body function. One primary action of glucocorticoids is to control their own secretion. Corticosterone can suppress stimulus mediated CRH secretion with a latency period of only seconds to minutes (35). Like CRH secretion, glucocorticoids inhibited PRL secretion through membrane associated nongenomic pathway. Glucocorticoids attenuate normal nocturnal peaks of PRL in humans and suppress vasoactive intestinal peptide-induced PRL release and intracellular cAMP levels within 30 minutes in rat pituitary cells. In tilapia pituitary cells, cortisol inhibited PRL release within 10-20 minutes (15,16,36). Glucocorticoids profoundly influence hepatic glucose metabolism *in vivo* and *in vitro*. In rat hepatocytes, dexamethasone reduces glycogen stores by directly affecting glycogen synthetase and phosphorylase activity (37). Investigations support a nongenomic glucocorticoid action on pigment redistribution, a phenomenon that might be crucial for the rapid color changes characteristic of many ectotherms. Dexamethasone caused rapid morphological changes, flattening of cell body and the expansion of dendrites in gold fish melanocytoma cells (38). From the literature reviewed above, it is obvious that glucocorticoids are involved in a number of nongenomic

responses at different levels of biological functions mostly in mammals, however, studies are meager in sub mammalian groups.

The well known effects of sex hormones are mediated through genomic mechanisms involving intracellular receptors. However, there is evidence that sex hormones can induce rapid responses through interaction with cell membrane and consequent changes in membrane function (21). An increasing body of evidence indicates that in several cell types of reproductive tissues, estrogens exert physiological effects that are too rapid to be mediated by the sequence of genomic activation. Pietras and Szego were the first to report the rapid effect of estrogen in 1975 (39). Rapid responses to estrogen were later noticed in preoptic septal and neostrial neurons (40), pituitary cells (41), and maturing human oocyte and granulosa cells (42,43). Low physiological levels of17â estradiol increased Ca^{2+} influx in enterocytes of female rat duodenum within 10 minutes (44). Increase of cAMP in response to estrogen in vascular smooth muscle cells, breast cancer, and uterine cells (45-47) are thought to be related to rapid estrogen-induced activation of membrane adenylate cyclase. Estrogen and other estrogenic compounds may exert rapid effects in the brain involving modulation of dopamine-induced excitatory responses and synaptic and other physiological functions (48,49). Synthetic estrogen, diethylstilbestrol (DES), significantly decreased gonadotrophin-stimulated 11-ketotestosterone productions in Atlantic croaker testes (50).

It is well recognised that 17β estradiol protects women against the development of cardiovascular diseases, such as atherosclerosis (51). The mechanisms of protection are many and represent actions of 17β estradiol at both the membrane and nuclear receptors. A variety of actions in CNS have been attributed to actions occurring at the plasma membrane. In several *in vitro* or *in vivo* models of experimental cerebral ischemia, 17β estradiol has preserved neurovascular endothelial cells and neurons (38, 52) .In human $P2X_7$ receptor 17β estradiol rapidly and reversibly inhibited whole cell receptor cation current (53). The support for the existence of distinct estrogen binding sites in the membrane came from the functional studies on genomic effects of 17β estradiol (54, 55). Estrogens have vasodilator actions involving both endothelium dependent (56) and endothelium independent mechanisms through a direct effect on smooth muscle cells (57). Estrogen rapidly increased serosal potassium transport in human colon 58) and prevents caspase-6-mediated neuronal cell death in human (59). Nongenomic estrogen-induced effects in most cases are likely to be mediated by a cell surface ER that is structurally related if not identical to the classic ER. However, as the spectrum of nongenomic actions is so diverse, more than one mechanisms of action have to be assumed, and more than one membrane receptors are likely to be involved.

The regulation by progesterone on myometrial contraction and related wider subjects of endocrinology of pregnancy and parturition has been reviewed several times during the last century. However, new evidence specifies that several progesterone metabolites and some synthetic steroids stimulate progesterone–like uterine relaxing effects (60). Progesterone has been shown to rapidly stimulate Cl^- (61) and Na^+ (62) fluxes in human spermatozoa. Besides these, a number of other rapid progesterone effects have been demonstrated, which include a dose dependent relaxation on rat saphenous artery (63), inhibition of contractile activity of murine jejunum (64), sodium absorption in Xenopus kidney (65), platelet aggregation in rat aorta (66),

The effects of androgens seem to be exactly similar to those of estrogens. A short-term administration of testosterone rapidly increased coronary blood flow in animal models (67). Rapid action of androgens is mainly concentrated on reproductive cells. Rapid effects of testosterone on intracellular Ca^{2+} concentration have been studied in human granulosa luteinizing cells. (68). In Sertoli cells, rapid elevation of cytosolic Ca^{2+} ion by testosterone and dihydrotestosterone was reported (69,70). These effects were also induced by testosterone-BSA conjugate, indicating a surface membrane effect (69). Testosterone was also known to induce intracellular Ca^{2+} ion in skeletal muscle cell cultures in rat (71). Testosterone inhibited arginine vasopressin release from hypothalamic slices in rat (72). Testosterone rapidly and possibly through nongenomic mechanism blocked the adenosine vasodilator effect and increased vasoresistance on isolated and perfused heart of rat (73). 5â dihydrotestosterone stimulated relaxation of rat aorta by acting directly on the membranes of smooth muscle cells (60). The androgen-induced increase of cellular cAMP levels may influence cell growth in the human prostrate cancer cell line through the intermediacy of sex hormone binding globulin (74). These effects were independent of sex and of the expression of classic androgen receptor (75). Testosterone inhibited chloride secretion in cultured rat efferent duct epithelia (76).

Rapid action of steroid hormones, vitamin D_3 and thyroid hormones on cellular signaling may be transmitted by specific cell membrane receptors. Although no receptor of this kind has been fully cloned up till now, binding sites in cell membranes have been characterized exposing binding features compatible with an involvement in rapid signaling. Characteristics of putative membrane receptors are completely different from those of intracellular steroid receptors (77). Such putative receptors are identified for almost all steroids. Estrogen membrane receptors are identified in pituitary cells of rat (78), in the uterus of rabbit (79) and in the testes of Atlantic croaker (50). Similar membrane receptors are also identified for glucocorticoids in amphibian brain (33,80-84), and in rat liver (85). However, the nature of GC membrane receptor is still a matter of debate. Considering the major achievements in this field, two principal candidates are suggested. On the one hand, membrane GC receptors in amphibian brain have been intensively studied that seem to be distinct from intracellular GR. On the other hand, data from mouse lymphoma cells suggest that membrane GC receptors are modified from intracellular GR (13). A membrane-associated testosterone receptor was identified in *Pseudomonas testosteroni* (86). Recently, testosterone receptor on cell surface of T cells and on IC-21 macrophages have detected by the use of confocal laser scanning microscopy and flow cytometry (87,88).

In addition to the proposed specific putative membrane receptors for steroids, recent studies on estrogen action indicate an involvement of the classical intracellular ER in rapid steroid action (78,79). These findings twisted the earlier concept that membrane receptors for steroids differ from the classical intracellular receptors. According to Caulin-Glaser *et al.*, 1997(89), this may be due to the possibility of locating some intracellular ER at cell surface. Recently, Falkenstein *et al.*, 2000 (13) have classified membrane receptors responsible for nongenomic action of steroids as follows. (1) Classical intracellular receptors identified for rat pituitary cells (78), rat hippocampal neuron (90), (2) Nonclassical steroid receptors without co-agonists. These types of receptors are identified for vitamin D (91,92), (3) Nonclassical steroid receptors with co agonists, identified mainly for neurosteroids (93,94), and (4) without any receptors (direct nongenomic action); this nonspecific steroid action can be expected at

non-physiological concentrations of steroids. This type of steroid action may occur by altering the physicochemical membrane properties such as the fluidity and microenvironment of membrane receptors (95,96). For a better understanding of nongenomic steroid action even in the clinical context, future research will have to target the cloning of membrane receptors for steroids and the evaluation of the clinical relevance of rapid steroid effects in general.

The rapid effects of steroid hormones are manifold. They involve plasma membrane binding, changes in membrane electrical activity, Ca^{2+} handling, G and Ras proteins, cAMP, cGMP, IP (3), DAG, phosphodiesterases, protein kinases, tyrosine kinases, ER kinases, and mitogen activated protein kinases (MAPKs) and nitric oxide synthase (97). In some cases these rapid actions of steroids are mediated through the classical steroid receptors that can also function as a ligand- activated transcription factor, whereas in other instances the evidence suggests that these rapid actions do not involve the classical steroid receptors.

Recently, Chen and Qiu in 1999 and 2001 (98,99) reported multiple signal transduction pathways for the rapid, nongenomic effects of glucocorticoids. Latest experimental results indicate that nongenomic effects of glucocorticoids are remarkably pleiotropic. A lot of observations indicated the potential involvement of G proteins (80), protein kinase A (15), protein kinase C (100) and mitogen activated protein kinase (101) pathways in evoking a nongenomic effect in these glucocorticoid treatments.

Latest classification of nongenomic action of steroids

The rapid effects of steroids have been grouped into different classes. But recently, Cato *et al.*, 2002 (3) have classified the rapid effects into three categories based on their mechanism of actions.

I. Nonreceptor mediated steroid actions at plasma membrane:

Steroids may modulate protein activity without first binding to a receptor to initiate a signaling cascade.

Progesterone	Rapidly & reversibly blocks voltage gated K^+ channels	Ehring *et al.*, 1998
Progesterone	Activate Ca^{2+} influx in human sperm cells	Blackmore *et al.*, 1990
Aldosterone	Enhance Ca^{2+} ions & cAMP in knock out mice	Haseroth *et al.*, 1999

II. Steroid effects through membrane associated receptors other than the classical steroid receptors:

The effects appear to be mediated by transmembrane receptors that are distinct from classical intracellular receptors

17β estradiol	Chicken granulosa cells	Morely *et al.*, 1992
17β estradiol	Splenic T cells	Benten *et al.*, 1999
17β estradiol	Osteoblasts	Lieberherr *et al.*, 1993
Androgen	Macrophages	Benten *et al.*, 1999
Androgen	Splenic T cells	Benten *et al.*, 1999
Androgen	Osteoblasts	Lieberherr *et al.*, 1994

III. Rapid effects occurring through membrane bound classical steroid receptors:

This is the most clearly defined rapid effect of steroids occurring through membrane bound classical steroid receptors. The membrane bound hormone binding entities were confirmed as classical steroid receptors by the use of antibodies directed to different regions of steroid receptors

ER a	Pappas *et al.*, 1994
ER a	Pappas *et al.*, 1995
GR	Gametchu *et al.*, 1991
GR	Gametchu *et al.*, 1991

Caveolae act as site for rapid action of steroids?

Caveolae are small plasma membrane vesicles most abundant in simple squamous epithelium. They may be flat, or invaginated and expressed singly or in bundle like clusters resembling grapes (102). These tiny vesicles are believed to act as sites for rapid action of steroid receptors. Both ER and AR have been identified in caveolae, where they interact with caveolin (a transmembrane phosphoprotein in caveolae) in a ligand dependent manner (103). One of the actions of steroid receptors thought to take place in caveolae is the estrogen-mediated rapid increase in endothelial nitric oxide synthetase activity leading to the production of nitric oxide in human endothelial cells (104).

Nongenomic action of steroids in teleosts

All the membrane effects of nongenomic action are focused primarily in mammals. This may be due to the pharmacological significance of nongenomic action of steroids in mammals. Till now, very little information on rapid action of steroids is reported in sub mammalian groups, particularly in teleosts. Recently, we have reported nongenomic action of steroids on branchial Na^+K^+ and Ca^{2+}ATPase's activity in a teleost (17,18) and nongenomic action of steroids on lipid metabolism in a vertebrate (19) for the first time. The nongenomic action of steroids on branchial Na^+K^+ATPase activity seemed to be mediated by Ca^{2+} ion in our study. We reported that treatment with 0.1µg / g body weight cortisol, corticosterone, testosterone and DES *in vivo* produced significant increase in the activity of branchial Na^+-K^+ ATPase and Ca^{2+} ATPase in *Oreochromis mossambicus*. The maximum activity for both Na^+-K^+ ATPase and Ca^{2+} ATPase was noticed after 30 or 60 minutes for all hormones. All the hormones significantly increased Na^+, K^+ and Ca^{2+} ions after 30 minutes *in vivo*. It was observed that 10^{-6} M cortisol, corticosterone and testosterone rapidly and specifically enhanced Na^+-K^+ ATPase as early as 5 minutes in the gill culture study. The activity of Na^+-K^+ ATPase in *vitro* was further increased at 10 minutes and the maximum activity was at 15 minutes. In the case of DES, 5minutes incubation did not produce any significant difference, while that of 10 minutes significantly stimulated Na^+-K^+ ATPase activity and the maximum activity was

at 15 minutes like that of testosterone. The rapid action of ATPase activity was not blocked by 0.1µg / g body weight actinomycin D *in vivo* and 10^{-6} M actinomycin D *in vitro* when treated prior to steroids. This action seems to be mediated through Ca^{2+} ion, involving a nongenomic pathway, although the physiological significance of such a pathway has not been clear. Cortisol and testosterone produced rapid and opposite effects on the lipogenic enzymes studied in the liver of *O. mossambicus*. Cortisol significantly decreased the activities of malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), as early as 5 minutes and isocitrate dehydrogenase (ICDH) as early as 10 minutes *in vitro* (10^{-6}M), and 30 minutes *in vivo* (0.1µg/g body weight) whereas the same doses of testosterone significantly stimulated the activity of all the enzymes as early as 5 minutes *in vitro* and 30 minutes *in vivo*. ActinomycinD treatment did not interfere with the inhibiting effect of cortisol on enzyme activities when measured at 10 min in the *in vitro* system. The transcriptional inhibitor appeared to partially block the effect of cortisol *in vivo*. The stimulatory effect of testosterone was insensitive to the action of actinomycin D both *in vivo* and *in vitro*. These effects appear to be brought about independently of new protein synthesis because the rapid responses occurred within a latent period of 5-30 minutes and were insensitive to the action of actinomycin D, suggesting a nongenomic action. The opposing action of cortisol and testosterone on lipogenic enzymes indicates that they are not only working in a catabolic and anabolic fashion, respectively, but also suggests their actions are specific. That is, the actions of cortisol are not one that would simply reflect an inhibition by any steroid, since testosterone had the opposite effect.

Cell membrane forms of steroid hormone receptors coupled to intracellular signaling pathways may also play an important role in hormone action. Membrane-initiated signals appear to be the primary response of the target cell to steroid hormones and may be prerequisite to subsequent genomic activation. Recent dramatic advances in the field of steroids' action at molecular level have established that cell surface forms of steroid hormone receptors coupled to intracellular signaling pathways may play a vital role. In view of recent developments, it may be assumed that membrane-initiated signals appear to be the primary response of the target cell to steroid hormones and may be prerequisite to subsequent genomic activation.

Frequent information on rapid steroid hormone effects in diverse cell types cannot be explained by the generally existing theory that centers on the activity of hormone receptors located exclusively in the cytoplasm and nucleus. Cell surface receptors of steroid hormone coupled to intracellular signaling pathways may also play a vital role in hormone action. Membrane-initiated signals appear to be the primary response of the target cell to steroid hormones and may be prerequisite to subsequent genomic activation. Recent dramatic advances in this area have intensified efforts to delineate the nature and biologic roles of all receptor molecules that function in steroid hormone-signaling pathways. Thus nongenomic mode of action of steroids has reflected profound implications for our understanding of steroid hormone actions in responsive cells and may lead to development of novel approaches for the treatment of many cell proliferative, metabolic, inflammatory, reproductive, cardiovascular, and neurologic diseases.

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