REVIEWS

The Target Cell Plasma Membrane and Steroid Hormones: the Beginning or End of the Debate?

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Drawing on experience gained in previous studies, the authors propose their own approach to defining the role the plasma membrane of the target cell plays in the mechanism of action of steroid hormones. The properties of membrane receptors for glucocorticoids on lymphoid cells are identified and examined, and possible ways in which the hormonal signal is transformed into a biological response of the target cell are described. The results provide a theoretical basis for the development of novel diagnostic techniques and pharmaceutical preparations.

Key Words: *plasma membrane; steroid hormones; receptors*

The introduction of radioligand analysis into research practice lent impetus to the active study of molecular mechanisms by which steroid hormones (SH) act. With the use of tritium-labeled steroids, proteins binding steroid molecules with high affinity and selectivity were discovered, first in liver homogenates and later in homogenates of other target organs for SH. Because hormone-binding proteins were first identified in the soluble fractions of homogenates, they were designated as **cytosolic receptors** for SH.

Further research into the physical and chemical properties of cytosolic receptors in the early 70s led to the emergence of a two-stage model of steroid action. According to the concept underlying this model, SH penetrate into the competent cell by simple diffusion and bind to cytosolic receptors. The resulting hormone-receptor complex is activated, acquiring a capacity to be translocated into the nucleus, where it initiates gene expression, this ultimately resulting in the synthesis of specific proteins that determine the cell's response to the hormonal signal. Thus, **the plasma membrane is assigned a passive role in this scheme.**

Following the advent of new investigative techniques and the development of a selectivity theory of drug action, we have been able to accumulate, over the past 10-15 years, a large body of experimental data on the molecular mechanisms of SH action.

The first debates concerning the role of the plasma membrane in SH action date to the mid-70s, when Pietras and Szego reported the discovery of binding sites for the naturally occurring hormone estradiol on the surface of myometrial cells [22]. The flurry of attention which this report provoked was due not only to the information it contained, but also to the novel methodologicy it described. Thus, 17β-estradiol molecules were covalently immobilized on capron threads, and these were then immersed in a myocyte suspension, incubated for 2 h, extracted from the suspension, and examined under an optical microscope: cells adsorbed onto the polymer were observed. When the pure carrier (without estradiol) was used or the biologically inactive optical isomer 17α-estradiol was immobilized, no cell absorption was in evidence - an indication that the effect

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recorded in the preceding experiment was specific. The downside of these exciting experiments was that the location of hormone binding sites could be evaluated only qualitatively; moreover, no attempt was made to analyze the biological significance of the phenomenon.

Later, classic preparative biochemical methods and radioligand analysis were employed to identify and quantify membrane steroid-binding sites, as a result of which specific binding sites for all classes of SH (estrogens, progestins, glucocorticoids, and mineralcorticoids) were localized in the competent cells [12,20]. Cytoplasmic membranes were found to contain two steroid-binding systems, one of which was characterized by saturation with and high affinity and specificity for tropic hormones and the other, on the contrary, by nonsaturation, low affinity and lack of specificity. Membrane preparations were subjected to various biochemical treatments to determine the chemical nature of these "recognition" systems. Preincubation of hepatocytes or their plasma membranes with the sulfhydryl-group blocker p-chloromercuribenzoate or with a proteolytic enzyme (pronase, papain) considerably reduced SH incorporation into the cells. It is significant that the steroid-binding capacity of cytosolic receptors remained unchanged under these circumstances [13]. There is no consensus regarding the biological role of the discovered specific SH-binding system. Some authorities, noting the low dissociation rate of hormone-receptor complexes, ascribe the function of hormone transport across the plasma membrane to steroid-bound membrane proteins, whereas others believe that this system comprises membrane-bound enzymes of steroid metabolism, in particular in the case of hepatic cell membranes.

The next stage in the debate between the advocates of the membrane step in the mechanism of SH action and followers of the classic scheme of steroid action was marked by mutual recrimination that the experiments were "contaminated". Indeed, it is not possible to obtain pure preparations of the membranous or cytosolic cell fractions with the available biochemical techniques. The degree of purification achieved, as estimated from the activities of marker enzymes for the plasma membrane (5'-nucleotidase) and cytoplasm (ketoso-1-phosphate aldolase) and defined as the ratio of specific enzyme activity in the final preparation to that in the original homogenate, ranges from 15- to 50-fold. Consequently, the presence of membrane binding sites for SH may be determined in part by an admixture of cytosolic fraction in the membrane preparation and vice versa.

It seemed that this controversy would be resolved by studies utilizing autoradiographic and immunohistochemical methods by which SH receptors can be visualized in whole cells. Such studies demonstrated the presence of both membranous and cytosolic hormone-receptor complexes. An unexpected finding was the discovery of unbound receptors for estrogens and progesterone in the nuclei of target cells. These results led to the development of three independent models of intracellular localization of SH receptors and fostered skepticism toward the data obtained with cell homogenates. In subsequent publications, limitations and artefacts inherent in the autoradiographic and immunohistochemical methods were revealed and analyzed. The functional role of membrane binding sites for SH remained undisclosed.

The approach proposed in our studies involves the use of polymeric derivatives of several naturally occurring and synthetic glucocorticoids (GC), including cortisone, prednisolone, and dexamethasone, as tools for localizing the SH receptor apparatus and for clarifying the molecular mechanism of its action [12]. As a hydrophilic macromolecular carrier (molecular weight of the order of 25,000 D), we chose poly-(N-vinyl-2-pyrrolidone), a compound used for medical purposes and regarded as biologically inert.

Our control experiments showed that copolymer molecules are structured and more compact in aqueous solutions than those of the carrier polymer because of hydrophobic interactions among the steroid fragments; such molecules show a high degree of hydrolytic stability under near-physiological conditions and do not penetrate into viable mammalian cells. Immobilization of an SH at position 21 of polyvinylpyrrolidone (PVP) does not

TABLE 1. Characteristics of Receptor Binding Sites for Glucocorticoids

Parameter	Membrane receptors	Intracellular receptors
Affinity, K_d	0.12–0.60 μM	5–30 nM
Receptor binding capacity	4–6 pmol/mg protein (65,000 binding sites per cell on average)	0.2–0.3 pmol/mg proteins (3000 sites per cell)
Relative affinity	cortisol ≈ progesterone > dexamethasone > estradiol > testosterone	triamcinolone > dexamethasone > cortisol > testosterone > progesterone > estradiol

alter the complex-forming activity of the original steroid vis-a-vis its biological molecular targets in model systems, such as serum albumin, transcortin (a specific carrier protein of blood plasma), and polyclonal antibodies to GC.

Over the past decade, studies using PVP-GC have generated a wealth of experimental evidence for the existence of specific GC binding sites on plasma membranes of various lymphoid cells including thymocytes, peripheral blood lymphocytes, bone marrow lymphoblasts, and plasmacytoma cells. Binding parameters and relative binding activities have been determined, mechanisms of transmembrane signal transmission have been explored, and their role in the formation of the final biological response of the cells has been defined. Taken together, our experimental data along with those reported by other authors have led us to conclude that **the specific binding sites for GC on the plasma membranes of lymphoid cells meet the main criteria established for receptors of biologically active substances; we have designated these sites as "plasma membrane receptors."**

Table 1 summarizes the main characteristics of membrane receptors for GC such as the parameters of specific binding K_d (equilibrium dissociation constant of the hormone-receptor complex) and maximal receptor binding capacity, and indicates the relative affinities of the receptors for various steroids [19]. It can be seen that the membrane and intracellular binding sites differ both in affinity for hormones and in density. Intracellular receptors have higher affinities (K_d of the order of 10 nM) and a much lower (approximately 30-fold) number of binding sites. These two receptor systems also differ in the ability to bind GC hormones and synthetic steroids. The synthetic GC dexamethasone and triamcinolone tend to bind to intracellular receptors (the K_d values of membrane and cytosolic receptors differ by a factor of 60 on average). The affinity of the naturally occurring GC cortisol and corticosterone for intracellular receptors is much lower (the ratio of their K_d values is only 4.2).

Until recently, the mechanisms of GC binding to membrane and to cytosolic receptors were considered separately. In our work, we used a variety of methodological approaches to devise a mathematical model that would allow for the interaction of GC with topologically distinct receptor systems and make it possible to predict their distribution in the cell and their effects.

We found that the distribution of naturally occurring GC between the membranous and cytosolic cell fractions is very different from that of synthetic GC [8]. For example, if the total concentration of a ligand such as dexamethasone is within 10 nM (i.e., within the region of physiological concentrations for GC), then virtually all of the dexamethasone is bound to intracellular receptors; to saturate membrane receptors with this steroid, its concentration has to be raised to 1000 nM or more. In contrast, cortisol in a concentration range of 0 to 10 nM is predominantly bound to membrane receptors, the number of its molecules associated with cytosolic receptors being small. Raising the total cortisol concentration to 400 nM results in saturation of both the membranous and intracellular systems with this steroid. The membranous receptor system thus acts as a buffer for naturally occurring GC. In the mechanism of action of synthetic GC, the membranous stage is curtailed.

Our experimental findings have been used to perfect a method for determining GC binding by lymphoblast receptors in patients with acute lymphoid leukemia.

Glucocorticoids are widely used in clinical practice for the treatment of malignancies, including acute lymphoblastic leukemia. However, the activity of GC preparations may be neutralized if the patient develops resistance to GC. In such cases hormones are no longer capable of inducing lysis of lymphoblasts and the hormonal therapy brings no benefit. Until now researchers have tried to interpret lymphoblast resistance as a consequence of abnormalities in the receptor apparatus of target cells, but have failed to demonstrate a consistent relationship between the body's susceptibility to GC therapy and the level of GC receptor binding. The apparent lack of such a relationship is due to the fact that lymphoblasts contain several types of GC receptors and acceptors differing in location, affinity, and density; hence the difficulties in determining the actual concentration of intracellular receptors for GC. Our studies have shown that in addition to an intracellular receptor system, bone marrow lymphoblasts contain SH-binding membrane sites which make a substantial contribution to the total specific binding of steroids [7]. Yet the schemes of radiological receptor analysis used so far disregard the importance of membrane binding sites for GC. We have developed a modified radioligand method for a differential evaluation of specific GC binding sites differing in location (membrane and intracellular sites) in lymphoblasts. Unlike the standard radioligand method which makes use of dexamethasone as a ligand of cytosolic receptors, the modified method is based on the use of dexamethasone in conjunction with cortisol, which is immobilized on PVP and to which

only membrane receptors for GC are accessible. Cortisol does not penetrate inside the cell and displaces dexamethasone from its membrane binding sites, with the result that dexamethasone predominantly interacts with intracellular receptors. Using the modified radioligand method, a latent form of acute leukemia accompanied by a fall in the concentration of intracellular GC receptors was identified in approximately 20% of cases undetectable by standard methods.

Above, we have tried to demonstrate that the use of a combination of steroids differing in membrane activity enables their efficacy to be altered in the desired direction.

Experimental data, including those from our laboratory, indicate that the "membranotropic" effects of SH whose triggering mechanisms are located on the plasma membrane are mediated by the system of intracellular second messengers. SH interaction with the plasma membranes of target cells alters the intracellular levels of cAMP and calcium ions [14,15].

It has been shown on various experimental models that GC elevate the basal cAMP concentration and are also capable of potentiating the action of other compounds activating adenylate cyclase. By a mechanism of competitive of synergistic activation, GC enhance the cAMP elevation induced by adenosine, epinephrine, isoproterenol, or prostaglandin E_1 [17]. On the other hand, GC have no effect on the cAMP elevation caused by sodium fluoride, a compound that differs fundamentally from other activators of adenylate cyclase in the mechanism of its stimulation. Unlike the above-mentioned endogenous biologically active substances whose effects are mediated by interaction with membrane receptors, NaF can directly activate regulatory GTP-binding proteins (G proteins) transmitting the signal to the catalytic subunit of the adenylate cyclase complex. These findings suggested that GC interact with G proteins directly. In the course of evolution, G proteins appeared only in animal cells (bacteria lack an intermediate link in the form of GTP-binding proteins), where they provide an additional level for the regulation of transmembrane signal transmission. Human peripheral blood lymphocytes treated with pertussis toxin, a selective G_i protein inhibitor, show a considerably reduced cAMP response to GC [9], which supports the above hypothesis. Further research, however, is required to define the precise role of G proteins in the mechanism of membranotropic SH action.

An important factor in the regulation of the interaction between the membrane receptor and adenylate cyclase is the mobility of cell membrane lipids. In order to rule out the nonspecific action of GC on cAMP levels, we assayed steroids for their effect on the microviscosity of target cell membranes by measuring the excimerization of a pyrene probe. The results led us to conclude that GC effects on the adenylate cyclase system are not mediated by alterations in the fluidity of plasma membrane lipids [16].

Elevation of the intracellular cAMP level is known to trigger a cascade of sequential biochemical reactions, the pivotal role in which is ascribed to specific cAMP-sensitive protein kinases (A kinases). Cytosolic receptors may be considered as a substrate for A kinases. The activation of cytosolic hormone-receptor complexes and their subsequent translocation to the nucleus are cAMP-dependent processes. As a consequence, the elevation of the cAMP concentration induced by GC at the plasma membrane level results in an alteration of their own intracellular receptors by a mechanism of homospecific regulation [5]. Here, membrane receptors act as modulators, preparing the cell to "perceive" the hormonal signal. The membranous and intracellular receptor systems are thus interrelated and probably regulated by a common mechanism. However, these two systems react in different ways to alterations in the level of endogenous corticosteroids . On days 5-7 after adrenalectomy in rats, their hepatocytes and lymphoid cells were found to have drastically decreased numbers of membrane receptors but substantially increased (1.5 to 2-fold) numbers of cytosolic receptors. Presumably, when the concentration of endogenous corticosteroids has decreased so that there is no longer any need for the buffer role of membrane receptors, the number of these is reduced by a negative feedback mechanism.

Another second messenger whose content is controlled by SH is calcium (Ca^{2+}). Unlike the action of estrogen, the action of GC on target cells is accompanied by a fall in the level of free calcium ions. GC inhibit in a dose-dependent manner (in the 0.1-5 μM concentration range) the mitogen-induced rise of Ca^{2+} in lymphoid cells and suppress the blast transformation reaction. The latter effect is specific (it is blocked by GC antagonists such as progesterone and dexamethasone-21-mesylate) and develops rapidly (within 30 min). Studies on the mechanisms by which GC influence calcium turnover in thymic lymphocytes, bone marrow lymphoblasts, and plasmacytoma cells have shown that steroids predominantly inhibit Ca^{2+} entry from the outside through the chemosensitive, "fast" calcium channels [1,3,4]. The liberation of

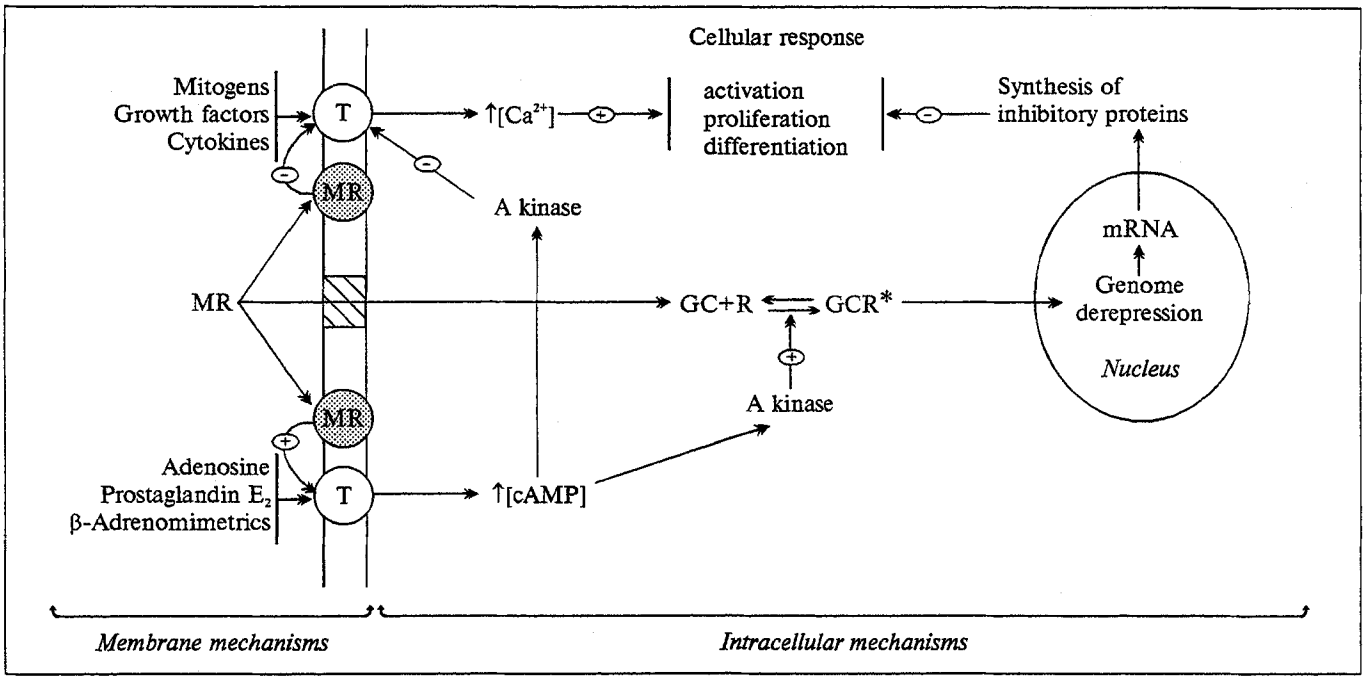

Fig. 1. Hypothetical scheme of the mechanisms by which glucocorticoids (GC) act on lymphoid cells. MR = membrane receptors; R = intracellular receptors; GCR* = activated form of the hormone–receptor complex; T = systems of transmembrane signal transduction involving second messengers; A kinase = cAMP–dependent protein kinase; + = activating influence; – = inhibitory influence. Hatched circles represent sites of specific GC binding on the plasma membrane.

calcium ions from their intracellular depots (endoplasmic reticulum vesicles) is less subject to the influence of GC. The GC effect on the formation of the second messenger inositol triphosphate is a delayed one, occurring 1 to 1.5 h after the start of exposure.

We used the calcium-blocking effect of cortisol on lymphocytes to evaluate the susceptibility of patients with asthma to GC therapy. Cortisol was found to inhibit the concanavalin A-induced elevation of Ca^{2+} levels in lymphocytes from a group of patients responding well to corticosteroid therapy, but it had an insignificant effect on Ca^{2+} levels in concanavalin A-treated lymphocytes from patients with a GC-resistant form of asthma. This method has been proposed for clinical use as a test for differentiating between hormone-resistant and hormone-susceptible forms of asthma [6].

The existence of SH receptors localized in the cytoplasm and on the cell membrane makes it feasible to separate the regulatory functions of SH at the different levels of cellular organization. Thus, the intracellular SH receptors mediate genomic effects of steroids such as the influence on mRNA synthesis, induction of programmed cell death (apoptosis), and stimulation or inhibition of the synthesis of tissue-specific proteins (e.g., inflammation mediators and cytokines, adrenergic receptors, and receptors for progestins and estrogens) [18]. The nongenomic, membrane-mediated effects include those on the turnover of second messengers, plasma membrane permeability for ions, transmembrane transport of amino acids, sugars, and nucleotides, and the release and metabolism of arachidonic acid products [10].

A logical continuation of research into the membrane-mediated, nongenomic effects of SH should be studies using anuclear cells such as erythrocytes and platelets.

The discovery of a membrane stage in GC action provided a theoretical basis for the search and synthesis of new GC preparations with selective actions on membrane receptors. To this end, two methodological approaches were used. In St. Petersburg, workers in the Pharmacology Department of the Pediatric Institute in collaboration with those at the Institute for High-Molecular Compounds have developed a novel method for the immobilization of known GC on hydrophilic polymers [2]. GC attached to a high-molecular carrier lose their capacity to penetrate inside cells so that the sites of their action are confined to cytoplasmic membranes of the target cells. The GC polyesters obtained are not inferior to the original compounds (dexamethasone and hydrocortisone) in terms of anti-inflammatory activity and are far superior to them in terms of anti-shock activity (in patients with traumatic shock). The effects mentioned above may be due not only to the interaction of the modified hormones with the membrane

receptors of target cells, but also to their elevated local concentrations. Another advantage of the GC polyesters is their much weaker adverse effects such as growth inhibition and thymic hypotrophy.

The other approach to designing "membranotropic" preparations was proposed by workers at the Center for Drug Chemistry at the Chemical-Pharmaceutical Institute in Moscow. A team headed by G. S. Gritsenko and M. E. Kaminka undertook a search for new compounds in the series of 16α-methylpregnane derivatives with selective local anti-inflammatory and antiallergic actions [11]. When used locally, they have proved to be far superior to dexamethasone, synaflan, and triamcinolone acetonide. Compounds of this series are free of several systemic side-effects such as those due to general catabolic activity, impaired carbohydrate and electrolyte catabolism, and inhibited hormonal function of the thymus and adrenals. Radioligand analyses have confirmed the high affinity of these preparations for membrane GC receptors; they bind to cytosolic GC receptors of the liver and thymus less efficiently.

Thus, the availability of GC preparations with a predominantly "membrane" type of action opens up new possibilities for rational hormonal therapy and diagnosis of several diseases.

Returning to the question posed in the title of this article, we may conclude that the plasma membrane does participate in the recognition of SH and in the subsequent transformation of the chemical signal into a biological response of the target cell. Because of this, **the membrane step in the action of steroids may be regarded as a distinct stage in the realization of their hormonal and pharmacological activities, supplementing the classic two-stage model of SH action** (Fig. 1).

We are adherents of the hypothesis that plasma membrane receptors exist and are the first to be involved in the "recognition" of tropic SH by cells. This does not mean that we eschew the debate on whether a nonspecific system of SH penetration into the cell exists. Indeed, one argument in favor of such a system is provided by our own experimental evidence for the presence of an unsaturable component in the plasma membrane. For the time being, however, we are powerless to analyze this kind of recognition. It is to be hoped that in time the mysteries surrounding SH interaction with the plasma membrane skeleton of the target cell will be unraveled with the aid of new methodology.

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