

Cross-talk in signal transduction

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The discovery of cross-talk between membrane-associated receptors and intracellular steroid and thyroid hormone receptors has gained much attention in recent years because of its multiple functional implications and biomedical significance. As the details of these interconnections between the two classic cascade mechanisms of hormone actions established, it will be of great use for various kinds of biomodulations. A greater action of steroid hormones can be achieved by using activators of membrane receptors together with a minimal dose of steroids which can avoid their toxic effects. It may be possible to activate a defective form of steroid receptors that may exist in various kinds of diseases.

COMMUNICATION by extracellular signals constitutes an important mode of regulation of gene expression and cellular metabolism. These signals (called ligands) are of varying chemical origin and act by interacting with their cognate receptors located either on cell surface membrane or in the cytosolic and/or nuclear compartments. The water-soluble signals (proteins, peptides, and neurotransmitters) bind to receptors located on the plasma membrane and trigger rapid changes in the activities of one or more enzymes involved in the cascade. On the other hand, lipophilic water insoluble signals (steroids, thyroid hormones, vit. D3 and retinoic acid) interact with their specific receptors located intracellularly. Among the lipophilic signals, the receptors for glucocorticoids are predominantly cytosolic^{1,2} whereas sex steroids, thyroid hormones, vit. D3, and retinoic acid bind to a member of nuclear receptor superfamily and modulate the expression of specific genes^{3,4}.

Protein and peptide hormone action

Protein and peptide hormones including neurotransmitters produce varying metabolic responses upon interacting with cell surface receptors. The binding of signals activates an enzyme that generates a short-lived increase in the concentration of an intracellular signalling molecule termed as second messenger. These second messengers may be 3',5'-cyclic AMP (cAMP), 3',5'-cyclic GMP (cGMP), 1,2-diacylglycerol (DAG), inositol 1,4,5-triphosphate (IP₃), and calcium (Ca²⁺). The elevated intracellular concentration of one or more such second messengers triggers a rapid change in the activity of specific enzymes or nonenzymatic proteins. Some other hormones like insulin do not produce a

known second messenger and act directly by modifying the activity of cytosolic proteins. Responses to the protein and peptide hormones including neurotransmitters are transduced by a protein whose activity is dependent on GTP/GDP binding. These signal transducing guanine nucleotide-binding regulatory proteins (G-proteins) are heterotrimeric proteins, localized in or at the inner face of plasma membranes^{5,6}. The function of these proteins is to couple receptors for extracellular signalling molecules to intracellular effector system^{7,8}.

The G-protein cycles between inactive GDP-bound and active GTP-bound forms. Activation is catalysed by the ligand binding to receptors and deactivation is

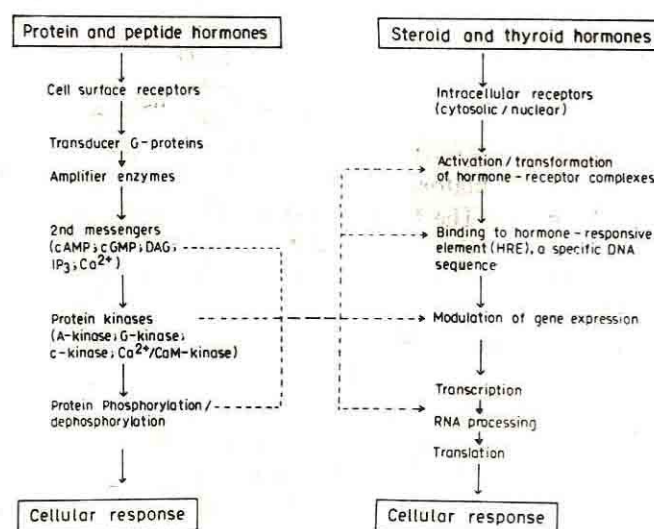


Figure 1. Sequence of events leading to the mechanism of protein/peptide and steroid and thyroid hormone action and their possible interaction. Left panel, protein and peptide hormones act by binding to specific cell surface receptor. The binding of such hormones activates enzyme(s) through G-protein and generates a short-lived increase in the concentration of intracellular signalling molecules termed as second messengers (cAMP; cGMP; DAG; IP₃; Ca²⁺). The elevated 2nd messengers trigger a rapid change in the activity of specific enzymes (protein kinases) which phosphorylate specific protein and elicit a cellular response. Right panel, steroid and thyroid hormones act by binding to specific intracellular receptor (cytosolic and/or nuclear). The hormone-receptor complexes undergo activation (transformation) and bind to a specific DNA sequence, termed as hormone-responsive element (HRE), generally upstream from the regulated gene. This interaction modulates the gene expression by changing the synthesis of mRNAs and respective proteins and produces cellular response. Dotted lines depict a possible interaction between protein/peptide and steroid and thyroid hormone action. The 2nd messengers and/or protein kinases may influence the steroid and thyroid hormone action at any of the possible steps like activation of hormone-receptor complexes and/or binding of these complexes to specific DNA sequences. Some of the phosphorylated proteins by protein/peptide hormone action may act as a *trans*-acting factor(s) and influence the gene expression in presence of steroid and thyroid hormones.

accomplished by intrinsic GTPase activity of the G-protein⁹. In the inactive form, G-protein exists as holo G-protein ($G\alpha, \beta, \gamma$) where GDP is bound to $G\alpha$. Agonist binding to specific cell surface receptor induces a conformational change in the α -subunit of G-protein, thereby reduces the affinity of G-protein for the bound GDP and causes subsequent dissociation of GDP from the G-protein¹⁰. This in turn increases the affinity of α -subunit to GTP¹¹. G-protein activation involves binding of GTP to $G\alpha$ and dissociation into $G\alpha$ -GTP and $\beta\gamma$ complexes. G-protein can interact with receptor again only when the subunits reassociate after the GTP is hydrolysed. Activation of G-protein leads to the modulation of an amplifier enzyme on the cytosolic face of the membrane. The amplifier enzyme adenylate cyclase converts adenosine triphosphate (ATP) into cAMP, whereas another amplifier phospholipase C cleaves the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). These second messengers induce cellular proteins to change their structure. It is accomplished by two well-characterized pathways. In one, the second messenger directly interacts with the effector protein and thus triggers a conformational change as in the case of Ca^{2+} binding to troponin C and/or calmodulin. In the other, the second messenger activates an enzyme called protein kinase which phosphorylates a specific protein. The phosphorylation induces a conformational change in the protein.

Two types of G-proteins are involved; one of them stimulatory (Gs) and the other one inhibitory (Gi)¹²⁻¹⁵. The stimulatory G-protein links itself to receptors called Rs. The binding of an external signal to such a receptor induces a conformational change in receptor. The change is transmitted through the cell membrane to Gs, making it susceptible to GTP, which comes from inside the cell. The binding of GTP to Gs confers Gs, an ability to activate adenylate cyclase which, in turn, converts ATP into cAMP. The information carried by the external signal is transmitted across the membrane and assigned to a second messenger. The activity of the Gs-GTP complex is ended by the hydrolysis of the bound GTP to GDP. The hydrolysis is catalysed by enzyme GTPase. The activity of GTPase is inhibited by the cholera toxin. The toxin thereby prolongs the life of the Gs-GTP complex, so that the cell produces cAMP continuously, even in absence of an external signal. In intestinal cells cAMP is a potent activator of fluid secretion which may lead to severe diarrhoea in patients of cholera. The other type of G-protein in this pathway mediates an inhibitory transduction. The interaction of an external signal to such receptor (Ri) brings a conformational change in the G-protein called Gi. This G-protein in turn inhibits adenylate cyclase. Pertussis toxin specifically blocks the activity of

inhibitory G-protein (for reviews see refs. 12, 14).

The second messengers have different cytosolic effectors through which they produce wide variety of cellular responses. cAMP pathway is mediated by a protein kinase (A-kinase) whose activity is dependent on cAMP. cAMP binds to the regulatory subunit so that catalytic subunit is free and active. This catalytically active protein kinase A phosphorylates several specific proteins. Hormones such as adrenaline (epinephrine) bind to receptors on the cell surface membrane, acting through G-proteins, influence adenylate cyclase (Ac), which makes cAMP. The cAMP stimulates an A-kinase, and the A-kinase activates lipase by phosphorylating the enzymes. In fat cells, the lipase initiates the mobilization of fat. Similarly, A-kinase also activates phosphorylase which catalyses the breakdown of glycogen in the liver. In addition to its role in activation of cellular enzymes, cAMP often causes an elevation of cytosolic level of another second messenger like Ca^{2+} ions. The calcium ions have several other divergent effects in modulating the conformation of many cellular proteins. Epinephrine has also been reported to act through cAMP to modulate the intracellular level of Ca^{2+} in muscle and nerve tissues¹⁶.

Some external signals have been found to stimulate the turnover of a membrane lipid and change the intracellular concentration of calcium. In particular, the phosphatidylinositol 4,5 bisphosphate (PIP_2) is hydrolysed into diacylglycerol (DAG) and inositol triphosphate (IP_3). The hydrolysis of PIP_2 upon binding of external signals to cell surface receptors is also coupled to G-protein activation. Activated G-protein modulates phospholipase C (PLC) which in turn hydrolyse PIP_2 into DAG and IP_3 (ref. 17). These metabolites have distinct role in the cellular regulation. IP_3 has been shown to mobilize stored Ca^{2+} from intracellular stores by binding to a specific receptor on the surface of endoplasmic reticulum and/or other intracellular vesicles that store Ca^{2+} (refs. 18, 19). This interaction induces a conformational change of specific Ca^{2+} channel proteins and allows Ca^{2+} ions to exit from the intracellular storage sites into the cytoplasm. Ca^{2+} ions in the cytoplasm have several roles to play in the cellular regulation. Hydrolysis of PIP_2 by PLC generates another important second messenger, DAG which activates an enzyme called protein kinase C (PKC). This enzyme is activated by Ca^{2+} and DAG together, suggesting an interconnection between these two second messengers. In the resting cell, PKC is an inactive, soluble cytosolic protein. Ca^{2+} ions allow it to bind to the cytosolic face of the plasma membrane, where it is activated by DAG. PKC plays a central role in the transduction of extracellular signals into a cellular response^{20,21}.

Steroid hormone action mechanism

Steroid hormones are lipid soluble and diffuse into cells where they bind to specific intracellular receptors. The hormone-receptor complexes then interact with specific DNA sequences usually located 100–300 bp upstream of a regulated gene^{3,4}. The steroid hormone receptors including thyroid hormones are a group of cell-specific *trans*-acting transcription regulatory factor whose activity is controlled by specific binding of the hormone^{3,4,22}. These ligand-activated transcription factors are thereby involved in the control of a wide variety of cellular processes in higher eukaryotes, including development, differentiation and ageing^{23–26}. The first intracellular receptor to be highly purified and shown to be a DNA-binding protein was for glucocorticoids produced mainly in the adrenal glomerular cortices. The interaction of this receptor with DNA was first studied using mouse mammary tumour virus (MMTV) DNA, transcription of which is enhanced in the presence of glucocorticoids^{27,28}. Using gene transfer experiments with the cloned proviral DNA, it is observed that the long terminal repeat (LTR) region has receptor-binding sites²². These regions (termed as glucocorticoid response element, GRE) also convey glucocorticoid-dependent induction upon a variety of linked heterologous promoters^{28,29}. DNAase I footprinting experiments have shown several binding sites for the purified glucocorticoid receptor within the hormone inducible segment of DNA^{22,30}. An analysis of GREs in the DNA of various genes yields a consensus sequence of 15 nucleotides (5'GGTACAnnnTGTCT-3')³¹. In genes that are negatively regulated by glucocorticoids, an imperfect copy of the GRE is found³¹.

A comparison of the sequence elements recognized by different steroid hormones constitutes a family of similar sequences. The responsive elements for glucocorticoids, mineralocorticoids, androgens and progesterone are identical whereas estrogen and thyroid hormone response elements have different consensus DNA sequences^{3,4}. The location of the steroid responsive elements (SREs) within regulated genes is quite variable, ranging from positions several kilobase pairs upstream of a promoter^{3,32} to a position within the first few hundred bp upstream³³ or downstream of the transcription start site³⁴. These SREs were demonstrated to behave as steroid-inducible enhancer sequences³². The exact mechanism by which steroid receptors increase transcriptional activity is not yet clearly elucidated. Several observations indicate that the presence of SRE alone is not sufficient for hormone inducibility but that, in addition, other regulatory elements are required³⁵. Multiple SREs occur frequently in the 5' flanking regions of hormone-responsive genes^{32,36}. When one of a pair of SREs is mutated or deleted, it decreases the inducibility of the adjacent

gene, suggesting that SREs act synergistically to achieve a high level of expression of hormone-responsive genes. The synergistic activation can result from cooperative binding of two or more *trans*-acting factors to their *cis*-acting elements presumably via specific protein-protein interaction^{37,38}.

With the advances in cloning techniques, the genes or the cDNA for several steroid hormone receptors have been cloned. Using sequence alignments, deletion studies, site-directed mutagenesis and domain-swap experiments, it has been shown that the receptor proteins consist of discrete functional domains^{39–41}. The carboxyl-terminus domain contains the hormone-binding site and a dimerization region. This domain is also involved in modulating gene expression. The amino-terminal domain has *trans*-activation and antigenic determinants. The central domain of the receptor has a sequence-specific DNA binding site and also nuclear localization signals². The DNA-binding domains of both the glucocorticoid and estrogen receptors have been determined using 2D NMR spectroscopy⁴². The two structures are very similar and represent a general means of sequence-specific protein-DNA recognition of a family of transcription factors. The central domain in the steroid and thyroid hormone receptors (comprising about 70–80 aa residues) is highly conserved amongst various steroid hormones^{3,4,39}. This region of the receptor polypeptide exhibits an array of cysteine residues in which two zinc atoms are tetrahedrally coordinated with four cysteines³. Most of the DNA binding *trans*-acting factors, including the above-mentioned ones, have 'zinc fingers'-like sequence motifs. These motifs help in proper recognition of specific DNA sequences in most of the DNA binding proteins⁴³. Upon proper alignment to specific DNA sequences, the hormone-receptor complexes modulate the expression of regulated genes.

Cross-talk in signal transduction

It is now being established that the hormone action on target cells is controlled not only by the concentration of the individual hormones and their cognate receptors alone as mentioned above rather the cascade mechanism of protein/peptide and steroid hormone actions may be interconnected and influenced by each other. Initial attempts in this direction were made by Katunuma *et al.*⁴⁴ and Sharma⁴⁵ using biomodulators of glucocorticoid action. Diacylglycerol (DAG), a potent activator of protein kinase C, is generated from membrane phospholipid by the action of phospholipase C (PLC), upon binding of signals to membrane receptors. DAG has been shown to enhance the induction of tyrosine aminotransferase (TAT) and ornithine decarboxylase (ODC) by dexamethasone, but

itself had no effect on these enzyme inductions in the absence of glucocorticoid⁴⁶. These findings speculated the involvement of protein kinase C in the expression of glucocorticoid action. To confirm further the role of protein kinase C in mediation of glucocorticoid action, it was shown that the induction of TAT by dexamethasone in rat hepatocytes was inhibited by 1-(5-isoquinolinesulphonyl)-2-methyl piperazine (H-7), an inhibitor of protein kinase C, but not by *N*-[2-(methylamino)ethyl]-5-isoquinoline sulphonamide (H-8), an inhibitor of cyclic nucleotide protein kinase⁴⁷. H-7 also inhibited the accumulation of glucocorticoid receptor complexes in the nuclear fraction of rat hepatocytes⁴⁷ and liver slices⁴⁸. Based on these findings, the involvement of protein kinase C was attributed to the translocation of glucocorticoid-receptor complexes in the nuclei^{47,48}.

Sphingosine, a selective inhibitor of protein kinase C, inhibits the induction of TAT and tryptophan oxygenase (TO) by dexamethasone in primary culture of rat hepatocytes⁴⁹. It did not inhibit the induction of TAT by dibutyryl-cAMP. These findings indicated that sphingosine, an endogenous modulator of protein kinase C, may influence the expression of glucocorticoid action. Sphingosine, the backbone moiety of complex sphingolipids is a potent and reversible natural inhibitor of protein kinase C activity *in vitro* and in cell systems and that the inhibition of protein kinase C requires the hydrophobic character and the positively charged amines^{50,51}.

Recently, protein kinase C has been implicated with the endogenous modulators of glucocorticoid receptor⁵². Modulators of glucocorticoid receptors are endogenous, water soluble, novel ether aminophosphoglycerides^{53,54}. These acidic cytoplasmic biomodulators also stabilize the steroid-binding ability of the hormone-free unactivated glucocorticoid receptors. Protein kinase C is a major signal transduction protein and is involved in the regulation of cellular growth, differentiation and tumour promotion^{20,21}. It is a Ca²⁺/phosphatidyl serine dependent ser/thr kinase which is activated by endogenous diacylglycerol. Various other compounds including sphingosine/sphingolipids have been reported to regulate protein kinase C activity both *in vivo* and *in vitro*^{50,51}. Because of the similarity in the structure of modulator of glucocorticoid receptor and the modulators of protein kinase C, it was predicted that these purified modulators of glucocorticoid receptor may regulate protein kinase C. It came true that the modulators stimulate purified protein kinase C activity in an enzyme cofactor-dependent manner⁵². These observations of the modulators regulating both glucocorticoid receptor function and protein kinase C activity *in vitro* suggest that the glucocorticoid receptor-mediated and protein kinase C-mediated signal trans-

duction pathways may be interconnected *in vivo*⁵². These findings support our view of potential cross-talk between glucocorticoid action and protein kinase C (refs. 44, 45).

In order to assign a definite functional site of protein kinase C influence in glucocorticoid action, we studied the role of protein kinase C inhibitor H-7 on the phosphorylation of glucocorticoid receptor. Glucocorticoid receptor is a phosphoprotein as are the other steroid receptors. Phosphorylation of receptors has been studied in a number of cells, tissues, and animals under a variety of conditions^{55,56}. However, the protein kinase(s) involved in phosphorylation of the steroid receptors has not been clearly identified, nor has the role of receptor phosphorylation in DNA binding and transcriptional modulation been elucidated. Although phosphorylation is widely studied, modification in regulating the activities of enzymes as well as non-enzymatic proteins, the conclusive evidence on the role of phosphorylation in regulating steroid receptor function is not clear. Phosphorylation and dephosphorylation of steroid receptors have been implicated with the receptor activation and also in the recycling of receptors^{41,56}. Using protein kinase C inhibitor (H-7) during *in vivo* phosphorylation of glucocorticoid receptors in rat liver slices, it has been shown that protein kinase C is not directly involved in phosphorylation of glucocorticoid receptor⁴⁸. Although, the possibility of site-specific phosphorylation of receptor protein with the same number of phosphate groups cannot be ruled out. Further experiments are needed to identify the protein(s) phosphorylated by protein kinase C which mediate the glucocorticoid action expression. The possibility of protein kinase C dependent phosphorylation of some other *trans*-acting factor(s) involved in glucocorticoid regulation of gene expression cannot be ruled out⁵⁷. It is now known that the activated glucocorticoid-receptor complex interacts with the AP-1 transcription factor in the nucleus⁵⁸⁻⁶⁰. AP-1 also binds to a DNA sequence that contains the phorbol ester response element and is thought to be activated by protein kinase C (refs. 58-60). These reports correlate an interrelationship between the steroid-hormone receptor-mediated signal transduction system and the membrane-hormone receptor cascade⁵².

Selected steroid hormone receptors can be activated in a ligand-independent manner by a membrane receptor agonist⁶¹. *In vitro*, dopamine mimics the effect of progesterone by causing a translocation of chicken progesterone receptor (cPR) from cytoplasm to nucleus. Dual activation of progesterone receptor by progesterone and dopamine was because of a serine residue which is essential for dopamine activation of receptor and not essential for progesterone-dependent activation⁶¹. Based on these observations, it is suggested that

steroid action mechanism may not be regulated exclusively by classical steroid receptor cascade but also modified by intracellular second messengers. In support of this observation, 8-bromo-cAMP has been demonstrated to mediate progesterone receptor-dependent transcription in the absence of progesterone⁶². This extends further the concept of inter-dependence of steroid receptors cascade to a membrane receptor cascade.

The cross-talk between membrane hormone receptor is not confined to the steroid hormone action rather it also occurs with thyroid hormone action. Thyroid hormone (triiodothyronine, T₃) dependent inductions of lipogenic enzymes (malic enzyme, fatty acid synthase and acetyl-Co A carboxylase) are inhibited by protein kinase inhibitors in chick embryo hepatocytes⁶³. These inhibitors themselves have no effect on enzymes in absence of thyroid hormones. They influence only when hepatocytes are treated with T₃. These findings corroborated with ours suggesting that on-going protein phosphorylation is required for stimulation of transcription of specific genes by steroid and thyroid hormones.

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