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Biochemistry of Transmembrane Signaling Mediated by Trimeric G Proteins

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Summary

Many extracellular signals are at the cell surface received by specific receptors, which upon activation transduce information to the appropriate cellular effector molecules *via* trimeric G proteins. The G protein-mediated cascades ultimately lead to the highly refined regulation of systems such as sensory perception, cell growth, and hormonal regulation. Transmembrane signaling may be seriously deranged in various pathophysiological conditions. Over the last two decades the major experimental effort of our group has been devoted to better understanding the molecular mechanisms underlying transmembrane signaling regulated by G proteins and to the closely related process of desensitization of hormone response. This review provides general information about the basic principles of G protein-regulated transmembrane signaling as well as about our contribution to the current progress in the field.

Key words

GPCR • Trimeric G proteins • Caveolae • Desensitization • Na, K-ATPase

Introduction

The extracellular signals – hormones, neurotransmitters and growth factors, bind to cell surface membrane receptors, which may be divided into the three main groups/families: i) coupled with GTP-binding regulatory proteins (GPCR), ii) ionic channels, and iii) tyrosine kinases. The hormone or neurotransmitter binding to stereospecific site (of the receptor) at the cell surface represents the first step in complicated sequence of molecular events transmitting the signal into the cell interior and initiating the ultimate physiological response. In G-protein-mediated pathway (Gilman 1987), the hormone/neurotransmitter binding induces conformational change of receptor molecule, which induces dissociation of trimeric G protein-complex (non-active) into the free (active) Gα and Gβγ subunits. Subsequently, both Gα and Gβγ activate the number of enzyme activities (effectors) or ionic channels which regulate the intracellular concentrations of secondary messengers such as cAMP, cGMP, diacylglycerol, IP_3, DAG, arachidonic acid, sodium, potassium or calcium cations.

Switching on and off the G protein cycle

The activation of receptor and dissociation of the non-active trimeric G protein complex into individual free (active) Gα and Gβγ subunits is quickly followed by switching off the signal transmission. This is caused by hormone (agonist)-induced activation of endogenous high-affinity GTPase located in the Gα subunit. This enzyme hydrolyzes the terminal γ-phosphate of Gα-GTP; the non-active Gα-GDP is formed. The Gα-GDP subunits than exhibit the high-affinity binding to the free Gβγ subunits and the non-active trimeric G protein complex Gαβγ is formed again. In this way, the G protein molecule is prepared for another round of activation/deactivation cycle. The activation/deactivation cycle of trimeric G proteins is very rapid process ranging from milliseconds (transducin) to seconds among different G proteins families (Stryer 1991).

Desensitization of hormone/neurotransmitter action

Besides the short-term switching on and off the signal initiated by agonist binding to GPCR, there are further types of dampening of the hormone response (Lefkowitz *et al.* 1980, Lefkowitz and Caron 1988, Benovic *et al.* 1988, O´Dowd *et al.* 1989, Hausdorff *et al.* 1990, Dohlman *et al.* 1991, Bourne and Stryer 1992, Collins *et al.* 1992, Lohse *et al.* 1993, Gershengorn 1994, Bohm *et al.* 1997). These mechanisms proceed in seconds to minutes time scale and are followed by even longer „negative feedback" regulatory loops proceeding within hours to days. The former type of desensitization is represented by receptor phosphorylation, sequestration and internalization. In this way, receptors are physically removed from cell membrane and transferred into the cell interior – *internalized*. As the adenylyl cyclase activity is never internalized (unpublished data from this and other laboratories), the receptor, *via* separation from its effector molecule, is transferred from the functional pool located at the cell surface (in the cell membrane) to an inactive pool located inside the cell. When present in the cell interior, GPCR are bound to small membrane vesicles (endosomes) which may recycle back to plasma membrane and renew the fully functional coupling with G protein, adenylyl cyclase and effectors. The last type of negative feedback regulation of GPCR action proceeds within hours to days. It is represented by *down-regulation* of receptor molecules. This is a terminal stage of receptor „life". The receptor protein is degraded/hydrolyzed in liposomes. In this way, the total number of receptors in the cell which has been stimulated for a very long time by respective agonist, is decreased.

As already mentioned, the short-term (seconds) hormone stimulation of GPCR results in desensitization of hormone response, which is primarily based on serine- or threonine-oriented phosphorylation at C-terminus of receptor protein, which is followed by an arrestin-induced functional uncoupling of receptor from G protein (Lefkowitz *et al.* 1980, Lefkowitz and Caron 1988, Benovic *et al.* 1988, O´Dowd *et al.* 1989, Dohlman *et al.* 1991, Bourne and Stryer 1992, Collins *et al.* 1992). Subsequently, the sequestration and internalization of receptors occurs within minutes. The agonist-induced internalization of GPCR proceeds almost exclusively *via* clathrin-coated vesicles (Tolbert and Lame 1996, Zhang *et al.* 1996).

G protein-based mechanisms of desensitization

Besides receptor-based mechanisms of desensitization of hormone response, there is another independent regulation, which is based on the changes in subcellular localization of trimeric G proteins. These mechanisms were revealed by studies of subcellular localization of G proteins in cells exposed for relatively long time (minutes to hours) to an agonist. The long-term stimulation is associated with internalization, solubilization and "down-regulation" of trimeric G proteins $G_s\alpha$ and $G_q\alpha/G_{11}\alpha$ (Ransnas *et al.* 1989, Svoboda and Milligan 1994, Milligan *et al.* 1995, Svoboda *et al.* 1996a, Kvapil *et al.* 1994, Novotný *et al.* 1995, Drmota *et al.* 1998, 1999, Svoboda and Novotný 2002). The internalization of $G_q\alpha/G_{11}\alpha$ proceeds independently from internalization of their cognate membrane receptor, TRH-R. The agonist-induced internalization of trimeric G proteins is not confined to $G_q\alpha/G_{11}\alpha$, but may be also demonstrated in other signaling cascades = β_2-adrenergic receptors and $G_s\alpha$ in S49 lymphoma cells (Ransnas *et al.* 1991, 1992, Yu and Razenick 2002). It seems likely, that G protein internalization and/or solubilization represent the general homeostatic mechanism protecting the target cells against excessive long-term stimulation. Conside-ring the generally accepted view that many GPCR act through the same class of G proteins, the internalization or solubilization of given G protein represents the basis for heterologous desensitization of hormone response.

Membrane domains/caveolae

The model cell lines expressing both G protein-coupled receptors (GPCR) and the cognate trimeric G proteins in high amounts exhibit dramatic agonist-induced transfer of G protein α subunits from plasma membrane to light-vesicular membrane fractions distinct from plasma membranes. This transfer was observed for M_1-muscarinic acetylcholine receptors and $G_q\alpha/G_{11}\alpha$ in CHO cells (Svoboda and Milligan 1994, Mullaney *et al.* 1996), for thyrotropin-releasing hormone receptor (TRH-R) and $G_q\alpha/G_{11}\alpha$ in HEK293 cells (Svoboda *et al.* 1996a) and for β_2-adrenergic receptors and $G_s\alpha$ in S49 lymphoma cells (Kvapil *et al.* 1994, Novotný *et al.* 1995). These cells also exhibit the solubilization and down-regulation (decrease in total cellular amount) of the cognate Gα subunits. The immunofluorescence studies indicate that under control conditions, i.e. without hormone, the localization of Gα is restricted exclusively to plasma membranes. This localization is unchanged up to 10-60 min of agonist stimulation; however, a fraction of Gα fluorescence signal is clustered to discrete segregated patches of fluorescent material when compared with the largely homogenous plasma membrane distribution prior to addition of the hormone (Drmota *et al.* 1998). This clustering phenomenon was observed as early as 10 min from the onset of agonist exposure and reminds the *"domain-like"* organization of plasma membranes. Further maintenance of an agonist (2 hours) resulted in a loss of plasma membrane-associated $G_{11}\alpha$ and appearance of distinct, punctuate staining with intracellular location of internalized G protein (*internalization*).

Internalization of GPCR (TRH-R) is a rapid process proceeding within minutes (Drmota *et al.* 1999, Novotný *et al.* 1999b), while the first internalization of $G_q\alpha/G_{11}\alpha$ is not detectable before 60 min of incubation with the hormone and is clearly demonstrated between 2 and 4 hours of exposure. TRH-R internalization proceeds *via* clathrin-dependent pathway unrelated to G protein (Nussenzveig *et al.* 1993, Ashworth *et al.* 1995, Gershengorn and Osman 1996, Petrou *et al.* 1997, Yu and Hinkle 1999), while the mechanism of G protein internalization is not known. Further analysis of these phenomena indicated that internalization of G protein is closely related to or mediated by "*membrane-domains/caveolae"*, the specific structural compartments of plasma membrane enriched with specific marker protein caveolin and other signaling molecules such as GPI-bound, peripheral membrane proteins and alkaline phosphatase (Pešanová *et al.* 1999). The membrane domains/caveolae are separated from bulk of plasma membranes with the help of detergent-extraction. Majority of plasma membranes are fully soluble in non-ionic detergents such as 1 % Triton X100 (60 min at 0 ºC) and therefore unable to float in density gradients when exposed to high-gravitational field. However, very small fraction of total membrane protein (less than 1 %) is resistant to detergent extraction and spontaneously forms the mixed detergent-lipid-protein micelles exhibiting low density. These *detergent-resistant* or *detergent-insensitive membrane domains/fragments* (DRMs or DIMs), when exposed to high-gravitational field in centrifuge and fractionated on sucrose density gradient float up (i.e. against the centrifugational force) into the areas of very low density corresponding to 15/20 % sucrose. Application of this isolation/fractionation procedure to cells which have been exposed to an agonist indicates a drastic depletion of the cognate G protein from DIMs/DRMs (Moravcová *et al.* 2004). The non-cognate (i.e. unstimulated) G proteins and other domain marker molecules are not decreased (Matoušek *et al.* 2003, 2004). Therefore, the membrane-domain organization of plasma membranes seems to represent an important structural determinant of the G protein pool relevant to desensitization of hormone action.

Beta-adrenergic receptors and $G_s\alpha$ signaling cascade in the heart muscle, brown and white adipose tissue

Among G protein-mediated signaling cascades, those initiated by β-adrenergic receptors have traditionally been the most frequently and best studied (Lefkowitz *et al.* 1980, Lefkowitz and Caron 1988, Benovic *et al.* 1988, O´Dowd *et al.* 1989, Dohlman *et al.* 1991, Kobilka 1992). Typically, β_1-adrenergic receptors play a crucial role in modulation of heart function. The typical β_1-adrenergic response of heart muscle to catecholamine stimulation is represented by increase in contractility (force of contraction), beat frequency and excitability (positive ino-, chrono- and bathmotrophic effect). Information from activated β_1-adrenergic receptors is transmitted through the stimulatory G proteins (G_s) to adenylyl cyclase and, after stimulation of this enzyme, intracellular concentration of cAMP is increased. Cardiac muscle contains a relatively high amount of $G_s\alpha$ protein compared to other tissues. To date,

nine isoforms of mammalian adenylyl cyclase have been identified (Patel *et al.* 2001), out of which type 5 and 6 are most abundant in the heart muscle (Espinasse *et al.* 1995).

When analyzing the β-adrenergic receptor-G_s-adenylyl cyclase stoichiometry, it has been realized that the individual components of this cascade are present in quite different amounts. Data collected from the model cell lines such as S49 lymphoma and NG108-15 cells indicated that adenylyl cyclase is quantitatively the least highly expressed component in this signaling pathway (Alousi *et al.* 1991, Kim *et al.* 1994). Similar data were obtained by Post *et al.* (1995) in isolated rat ventricular myocytes. The quantitative ratio between different members of β-adrenergic receptor initiated cascade in heart muscle cells was astonishing – 2.1×10^5 β-adrenergic receptors, 4.7×10^7 $G_s\alpha$ molecules and only 6×10^5 of activated adenylyl cyclase molecules. Quantitation of the $G_s\alpha$/adenylyl cyclase complexes was performed by measurement of the number of high-affinity [^{3}H]forskolin binding sites = about 6×10^5 per cell (Post *et al.* 1995). These results demonstrated very convincingly that under physiological conditions, $G_s\alpha$ is present in high excess relatively to either receptor or active effector sites. Since the single receptor may activate multiple G protein molecules, availability of adenylyl cyclase is likely to represent the rate-limiting step of the whole cascade, e.g. in hormone-induced stimulation of cAMP and muscle contraction.

The myocardial β_1-adrenergic response and β_1-receptor/G protein/effector (adenylyl cyclase) coupling may be significantly altered by a number of endogenous and exogenous regulatory factors, which affect this organ under various physiological and pathophysiological conditions. Among them, early phases of postnatal development and maturation, thyroid hormones, pressure-induced overload and hypoxia have been considered in our recent experimentation.

Our study analyzing the effect of thyroid status on the β-adrenergic receptor-mediated signaling in the developing rat myocardium showed that hypothyroidism in immature rats was associated with markedly reduced myocardial β-adrenoceptor density, lower content of $G_s\alpha$-L (the long isoform of $G_s\alpha$) and increased amount of $G_i\alpha 2$ and $G_i\alpha 3$ proteins. These changes were accompanied by substantially diminished sensitivity to the inotropic effect of isoproterenol (Novotný *et al.* 1999a). On the other hand, no change in β-adrenoceptor number, an increased level of $G_s\alpha$-L and decreased level of $G_i\alpha 2$ were found in hyperthyroid myocardium. Altered thyroid status also affected the membrane/cytosol balance of some G protein subunits in neonatal rat myocardium (Novotný *et al.* 2001). Hence, physiological levels of thyroid hormones are an important modulator of the normal maturation of the β-adrenergic signaling system in the developing rat heart. G protein-coupled signaling pathway appears to play an important role in the development of cardiac hypertrophy and its progression to heart failure. Our recent data also indicated that hypertrophy induced by sustained pressure overload does not affect the G protein distribution in newborn rats, but appreciably reduces the content of $G_s\alpha$-L in myocardium from adult animals. The concomitant derangement of the adenylyl cyclase signaling observed in immature as well as in adult rat myocardium was reflected by severe cardiac dysfunction (Novotný *et al.* 2003). We have also investigated the myocardial adenylyl cyclase signaling complex in rats exposed to chronic high-altitude hypoxia. Adaptation to hypoxia did not influence the number of β-adrenoceptors and the content of predominantly membrane-bound $G_s\alpha$, but it raised the amount of cytosolic $G_s\alpha$ in right ventricles (Hrbasová *et al.* 2003). Activity of myocardial adenylyl cyclase as well as functional activity of $G_s\alpha$ was significantly lower in chronically hypoxic rats and alterations in cardiac adenylyl cyclase system were only partially reversible after a 5-week recovery of animals at normoxia.

The typical β_1-adrenergic receptors were identified in *brown adipose tissue* (BAT) where they co-exist with the β_3-type (Svoboda *et al.* 1979, 1984a,b,c, Svartengren *et al.* 1982, 1984). The two types of receptors are periodically switched on and off when needed either to elicit the growth and differentiation of this tissue, or, in fully differentiated cells, to induce the highly specialized physiological response – thermogenesis (Cannon and Nedergaard 1985, Cannon *et al.* 1996a,b, Nedergaard *et al.* 1996). In intact brown adipose tissue, the main function of β_3-receptors is the induction of acute lipolytic, respiratory and thermogenic response. This type of response was also demonstrated in isolated brown adipocytes. The transmission through the β_3-adrenergic pathway, similarly to β_1, is realized mainly through activation of $G_s\alpha$ protein, but $G_i\alpha$ and Gβ subunits also participate (Svoboda *et al.* 1993, 1996b). The involvement of $G_i\alpha$ seems to be the main difference from the typical β_1-cascade. G proteins of $G_q\alpha/G_{11}\alpha$ family were also identified in brown fat (Bouřová *et al.* 1999a,b), but their physiological role is less understood.

It is probably related to the presence of α-adrenergic signaling pathway in this tissue. When judged from the quantitative characteristics of an overall physiological response, the significance of α-adrenergic receptors, $G_q\alpha/G_{11}\alpha$ proteins and of calcium-mediated signaling in brown fat, is, however, of minor importance.

When cultivated in tissue culture, pre-adipocytes isolated from young mice differentiate into typical brown adipocytes characterized by multilocular fat depots, full respiratory response to norepinephrine and expression of specific marker, an uncoupling protein of inner mitochondrial membrane – UCP. The analysis of individual isoforms (variants) of $G_s\alpha$ protein by immunoblot technique and correlation with the ability of isoprenaline and other β-adrenergic agonists to stimulate adenylyl cyclase in isolated membrane preparations indicated that the differentiation of brown adipose tissue is associated with preferential expression of the short variant of $G_s\alpha$, $G_s\alpha S$ (Bouřová *et al.* 2000). Therefore, the expression of $G_s\alpha S$ seems to be related to the generation of typical β_3-adrenergic responsiveness/phenotype. The differences in functional activity between two variants of $G_s\alpha$, $G_s\alpha L$ and $G_s\alpha S$, have also been noticed and analyzed in details in other cellular systems (Seifert *et al.* 1998).

An effort to affect or regulate the over all energy balance of the whole mammalian organism was oriented to the detailed analysis of metabolism of white adipose tissue (Kopecký *et al.* 1996). An over-expression of prototypical protein of fully differentiated brown adipose tissue, an uncoupling protein of inner mitochondrial membrane – UCP1, was achieved selectively in white adipose tissue of transgenic mouse (aP2-*Ucp*1). These animals are resistant to the development of obesity induced by genetic or dietary factors, reflecting a lower accumulation of triacylglycerols in all fat depots except in gonadal (epididymal) fat. Quantitative detection of G_s and G_i proteins in transgenic mouse indicated increased content of $G_s\alpha$ and decreased content of $G_i\alpha$ proteins (Flachs *et al.* 2002). Thus, the complement of trimeric G proteins is altered fully in line with the expected increase in capacity of lipolytic response to catecholamines (which is, however, not the case). Respiratory response of isolated adipocytes to noradrenaline is decreased. This surprising result seems to be caused by complicated set of mutually interrelated negative (feedback) loops operating under physiological conditions. Low ATP/ADP levels inhibit lipolytic response to catecholamines and represent the characteristic feature of "UCP transgenic state" of white fat. Phenomenologically, this type of paradoxical effect reminds desensitization of hormone-responsiveness of brown adipocytes isolated from cold-acclimated animals (Svartengren *et al.* 1982, Svoboda *et al.* 1984a,b,c, 1993, 1996b).

The G protein-mediated signaling in brain (G_i and G_o proteins)

The two above mentioned examples of GPCR were taken from typical G_s mediated cascades, i.e. those mediated by cholera-toxin sensitive, adenylyl cyclase stimulating G proteins. The second main type of G proteins is represented by those inhibiting adenylyl cyclase in pertussis-toxin sensitive manner (G_i1, G_i2, G_i2, G_o1, G_o2). These G proteins are present in large quantities in the brain where they inhibit adenylyl cyclase activity and modulate of numerous ionic channels (Giershik *et al.* 1986, Goldsmith *et al.* 1987). Similarly to heart muscle, the ability of G_i/G_o proteins to affect adenylyl cyclase (AC) in positive or negative manner is dramatically altered in the course of brain development. Around postnatal day 12, marked activation of G-protein/adenylyl cyclase coupling is observed which disappears with further maturation (Ihnatovych *et al.* 2001, 2002a,b, Hejnová *et al.* 2002). The reason for this decrease is unknown because the catalytical site of AC as well as the amount of different types of AC and G proteins is unchanged or increased. Furthermore, this decrease cannot be explained by the changes in respective receptor number or affinity ($GABA_B$, opioid, adrenergic) as the number and affinity of these receptors towards specific radioligands is unchanged or even increased. Therefore, at this step, one has to consider the newly discovered set of regulatory molecules, called regulators of G protein signaling (RGS), which are loosely bound to plasma membrane and therefore probably lost in the course of biochemical analysis. It will be the aim of our future studies to understand the mechanism of RGS binding to subcellular membrane fractions derived from the brain and to refine the experimental conditions (both in the terms of subcellular fractionation studies and correlation with the *in vivo* data) which would allow to study the activity of these proteins in natural tissues. Preliminary data on transfected cell lines suggest that reversible palmitoylation of RGS plays a crucial role in RGS-membrane interaction. The integrity of RGS binding to membrane may be therefore drastically altered by changing the redox state of isolation medium.

Regulators of G protein signaling

Regulators of G protein signaling (RGS) are multifunctional signaling proteins that are responsible for modulation and integration of G protein signaling pathways. More than 30 members of RGS family share a conserved RGS domain which binds directly to activated Gα-GTP subunits. RGS then act as GTPase activating proteins (GAPs) and limit the life-time of GTP bound to Gα during the G protein activation/deactivation cycle. This leads to the inhibition of G protein signaling (Hollinger and Hepler 2002).

The existence of regulatory factors increasing the rate of GTP hydrolysis had to be assumed when the rates of GTPase activity of isolated Gα subunits in reconstituted systems with purified components (without RGS proteins) were determined. The G protein-mediated signaling like phototransduction or stimulation of ionic channels was too fast to be realized by slow endogenous GTPase of isolated Gα subunits (Arshavsky *et al.* 2002). It was suggested that there must exist some factors accelerating the rate G protein deactivation and, subsequently, the RGS proteins were identified to be responsible for this process. Individual RGS proteins associate with different Gα subunits of all the main classes of trimeric G proteins. The classification of RGS into six distinct groups (RZ, R4, R7, R12, RA and RL) comes out from structural and functional similarities within these subfamilies. Whereas almost all members of RZ and R4 family (e.g. Ret-RGS1, RGS1-5) are small protein molecules representing just a little more than RGS domain itself and serve mainly as GAPs for trimeric G proteins, RGS proteins from R7, R12, RA and RL subfamilies (e.g. RGS7, RGS9, AKAP2, GRK2 and p115RhoGEF) exhibit rather complicated structure endowed with other domains overlapping among different classes of RGS. Thus, RGS proteins have to be viewed as multifunctional molecules (i.e. both regulatory and signaling) of crucial importance in the modulation of G protein signal transduction. RGS represent a "missing link" between *in vitro* biochemical data in reconstituted systems and fully functional signaling cascades working under physiological or pathophysiological conditions in a living cell. Considering recently published review articles in this area (Burchett 2000, Hollinger and Hepler 2002), RGS-oriented research provides the new topic of interest for general as well as cellular physiology.

Receptor for cardiac glycosides: Na,K-ATPase

Numerous enzyme activities are located in the cell surface membrane. Besides those directly involved in hormone action such as adenylyl cyclase, others participate in more distal steps of hormone-initiated cascade modulating the ultimate physiological response by complicated feedback loops fitting the metabolism of target cells to the optimal physiological response. The sodium plus potassium-activated, magnesium-dependent, ouabain-inhibited adenosine triphosphatase, E.C. 3.6.1.3 (Na, K-ATPase), which is the plasma membrane enzyme catalyzing the active transport of sodium and potassium across the cell membrane, represents the receptor for cardiac glycosides, group of potent toxins with a strong effect on the heart muscle. Ouabain and other cardiac glycosides selectively bind to this enzyme molecule, block its activity and, by increasing intracellular sodium and calcium levels, they elicit strong positive inotropic effect on heart muscle (myocardium).

Studies of Na,K-ATPase were one of the crucial subjects followed in our laboratory for a relatively long period of time. Originally, the effect of catecholamines on this enzyme activity was tested in order to explain the antilipolytic effect of ouabain on catecholamine (adrenaline)-induced lipolysis in white adipose tissue. Early studies of Mosinger (1969, 1972) indicated that about 30 % of adrenaline-induced lipolysis was blocked by ouabain or by depletion of potassium from the extracellular medium. Both these experimental conditions suggested Na,K-ATPase as a potential site of catecholamine action. When studying the direct effect of catecholamines on Na,K-ATPase in brain (cerebral cortex) and other tissues, we were surprised to find that this enzyme was drastically inhibited by endogenous factors present in cytosol (soluble) fraction, supernatant 100,000 x g. This inhibition correlated with manifestation of lipoperoxidative damage induced by ascorbic acid and traces of transitional metals such as iron. Catecholamines were able to protect Na,K-ATPase against lipoperoxidative damage which was quantitatively measured as malonyldialdehyde production. The anti-peroxidative action of catecholamines was based on their orto-catechol structure which was able to bind metal into the strong chelate. The chelated form of metal was unable to induce lipoperoxidation which is represented by complicated sequence of radical reactions proceeding in the presence of oxygen and leading to breakdown of

double bonds in aliphatic chain of unsaturated fatty acids (Svoboda and Mosinger 1981a,b, Svoboda *et al.* 1986).

These original studies were later extended to analysis of vanadyl (VO_2^+)-, vanadate (VO_3^-)- and phospholipase A_2-induced inhibition (Svoboda *et al.* 1984d,e, 1988), to demonstration of essential role of membrane lipids containing the unsaturated fatty acids such as arachidonate in the regulation of Na,K-ATPase activity (Vyskočil *et al.* 1983, 1987, Svoboda *et al.* 1988) and to distinction between dynamic and structural (lipid order) characteristics of biophysical state of membrane affecting the catalytical cycle of Na,K-ATPase (Amler *et al.* 1987, 1988a,b). The two types of ouabain binding sites were distinguished on the basis of their different sensitivity to monovalent cations, phospholipase A_2 and unsaturated fatty acids. Detailed chemical modification studies were performed with the aim to compare the action of numerous site-oriented reagents on catalytical, ATP-binding site with their effect on receptor (ouabain)-binding site (Teisinger *et al.* 1992). With the few exceptions, the inhibition of one site induced simultaneous inhibition of the other. Therefore, the information about inhibition, i.e. conformational change induced by an inhibitor, had to be transmitted *via* "long-range order" interactions from one site to another. When comparing the results obtained from *in vitro* studies of brain microsomal membranes or isolated enzyme (biochemical assays) with *in vivo* measurements of electrogenic sodium pump (Zemková *et al.* 1985, Teisinger *et al.* 1992), it might be concluded that inhibition of catalytical site of Na,K-ATPase located at the inner surface of plasma membrane was transmitted along the transmembrane segments to the ouabain-binding site located at the outer surface of plasma membrane. The site-oriented chemical reagents tested in these experiments were N-ethyl maleinimide, mersalyl and p-chlormercuribenzoate (SH-groups); acetic anhydride, pyridoxal-5-phosphate, 1-fluoro-2,4-dinitro-benzene (DNFB), N-ethoxy carbonyl-2-ethoxy-1,2-quinoline (EEDQ), 4,4-diisothiocyanostilbene-2,2-disulphonic acid (DIDS), 1-ethyl-3,3-dimethyl amino-propyl-carbodiimide (EDC), Woodward reagent (amino and carboxy groups), N-bromosuccinimide, N-chloro-succinimide, Koshland reagent and diethylpyrocarbonate (tryptophane, tyrosine, histidine) and 7-chloro-4-nitrobenzoxy-1,3-diazole (NBDCl), tetranitromethane (TNM), N-acetylimidazole, 2,3-butanedione, phenyl-glyoxal (tyrosine and arginine). In order to estimate directly the conformational changes of Na,K-ATPase molecule, our studies also included the site-oriented fluorescent probes such as eosine maleimide, fluorescein isothiocyanate and biotin maleimide (Hakaufová 1990).

Chemical modification studies further indicated that functional coupling of various cysteine residues (present in α subunit of Na,K-ATPase molecule) to the catalytical, ATP-binding site was not the same (Teisinger, Hakaufová, Svoboda, unpublished data). Similar situation existed for arginine, tyrosine and histidine residues. The dose-response curves of Na,K-ATPase inhibition induced by a given site-oriented reagent exhibited multiple affinities consistent with the view that multiple amino acids affect the catalytical, ATP-site with different affinities. Therefore, it was an inherent lack of specificity in action of any of these substances which precluded us to define clearly the significance of a given amino acid in either catalytical or receptor binding characteristics of Na,K-ATPase molecule. Therefore, site-directed mutagenesis and other genetic manipulations of Na,K-ATPase structure were introduced and detailed structure-function correlation studies of this enzyme molecule were carried out. When introducing these modern techniques, detailed mapping of ATP-binding site present in large cytoplasmic domain was performed and essential participating amino acids of the Na,K-ATPase were identified (Ettrich *et al.* 2001, Kubala *et al.* 2002, 2003, Krumscheid *et al.* 2003, Hofbauerová *et al.* 2003). The later studies were performed in collaboration with the Laboratory of Protein Structures headed by Dr. E. Amler.

Conclusions

Understanding the detailed mechanism of membrane receptor / G protein / effector coupling, the changes in cellular localization of these signaling molecules when affected by hormonal or neurotransmitter stimulation and the relationship between structure and function of receptor molecule when existing in plasma membrane are some of the crucial questions of modern physiology and molecular pharmacology. The answers to these questions are necessary prerequisites for understanding of any physiological process or function. This statement may be clearly documented by dynamic development of research oriented to membrane receptors in general as well as by the simple fact, that more than 80 % of the new drugs introduced into the medical practice in recent years was designed on the basis of structure/function correlation studies of G protein-coupled receptors.

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