

**Short Thesis for the degree of doctor of philosophy (PhD)**

**Correlations between the dipole potential, ErbB receptors and  
lipid rafts**

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## **Correlations between the dipole potential, ErbB receptors and lipid rafts**

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The Examination takes place at the Seminar Room F.402. of the Life Science Building, Faculty of Medicine, University of Debrecen, at 11:00 AM, April 12, 2018.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:00 PM, April 12, 2018.

## 1. Introduction

### 1.1. The dipole potential of the cell membrane

The eukaryotic cell membrane is a highly complex structure because of its lateral heterogeneity, the presence of membrane microdomains, and the “trinity” of membrane potentials, including transmembrane, surface, and dipole potentials. The least known, the dipole potential originates from the preferential alignment of interfacial water dipoles and dipolar segments of phospholipid and sterol molecules. Due to the arrangement of molecular dipoles, the interior part of the bilayer is characterized by a large positive potential with a magnitude of several hundred millivolts, resulting in a dipole electric field much stronger ( $10^8$ - $10^9$  V/m) than that of the other two membrane potentials.

Because of this large electric field, the dipole potential influences the conformation and the function of membrane proteins through effects exerted on their transmembrane domains (TMD). Consistently, significant effects of the dipole potential on the structure and function of proteins have been demonstrated in bacterial ionophores, voltage gated ion channels,  $\text{Na}^+/\text{K}^+$  ATPases, P-glycoproteins and G-protein coupled receptors.

The magnitude of the dipole potential is mainly determined by the lipid composition of the cell membrane, as it is influenced by whether the hydrophobic chains of phospholipids contain ether or ester bonds; the presence of double bonds in phospholipid chain regions; and the amount of sterols in the membrane. Its magnitude can be decreased experimentally by using phloretin, and increased with 6-ketocholestanol.

Because the dipole potential is entirely located within the low dielectric, hydrophobic interior of the plasma membrane, it is difficult to measure directly. Although several methods have been published to estimate the dipole potential, including measurement of the permeability of large hydrophobic ions, cryoelectron microscopy, molecular dynamics simulations, atomic force microscopy, and vibrational Stark effect spectroscopy, the application of voltage sensitive fluorophores represents the most widely accepted tool. Among these, the most commonly used molecules are the aminostyrylpyridinium derivatives, particularly di-8-ANEPPS. The applicability of this fluorophore is based on electrochromism (Stark effect), i.e. spectral shifts in response to changes in the magnitude of the local intramembrane electric field. Di-8-ANEPPS is mainly applied in an excitation ratiometric assay, when after exciting the dye at two different wavelengths corresponding to the two opposite edges of its excitation spectrum, the two emitted fluorescence intensities are measured and their ratio is calculated, which was shown to depend mostly on the magnitude of the membrane dipole potential. Under certain circumstances the use of another independent method might be beneficial when measuring the dipole potential.<sup>3-</sup>

hydroxyflavone derivatives are good candidates in these cases due to dramatic changes in their emission spectra as a function of the intramembrane electric field. These fluorophores are characterized by two well-separated bands in their emission spectra belonging to normal (N\*) and tautomer (T\*) excited states of their flavone chromophore. Species T\* appears as a result of an excited-state intramolecular proton transfer (ESIPT) reaction that is very sensitive to changes in the local electric field. The relative intensities of the N\* and T\* fluorescence emission bands can be used to measure the dipole potential of the cell membrane. Various 3-hydroxyflavone derivatives have been designed in which the chromophore is oriented in opposite directions with respect to the bilayer plane. Consistently, the N\*:T\* emission ratio of PPZ8 and of its related analog, F66, change in opposite directions upon modifying the dipole potential. Their simultaneous application is especially beneficial, as in the case of an alteration of another membrane biophysical parameter opposite changes in the spectra of the two fluorophores are expected. Thus, changes in the dipole potential can be differentiated from the alteration of other parameters.

## **1.2. The ErbB protein family**

Members of the epidermal growth factor receptor family, the ErbB proteins (ErbB1=EGFR=HER1, ErbB2=HER2/Neu, ErbB3=HER3, ErbB4=HER4) belong to the group of receptor tyrosine kinases and are important in early embryogenesis, in the development of the central and peripheral nervous system, various glands, heart muscle and cardiac valves. Since their activation stimulates signaling processes leading to cell division, they play important roles in the pathogenesis of different tumors (breast, lung, ovarium, cervix, gastrointestinal carcinomas, glial tumors), as their mutation or overexpression may result in transformation of the cells.

The ErbB proteins possess an extracellular ligand binding, a transmembrane and an intraceullular kinase domain. Besides their role in tumorigenesis, the major scientific interest in this receptor family stems from the fact that they are prototypes of dimerization-activated proteins. According to the classical allosteric activation model, in their resting state the receptors are found as monomers in the cell membrane. Binding of a ligand (for example EGF) induces a conformational change of the extracellular domain (ECD), leading to the homo- or heterodimerization of the receptor monomers, which results in the activation of the neighboring cytoplasmic tyrosine kinase domains through receptor-receptor interactions. This increased kinase activity leads to the (cross)autophosphorylation of receptor monomers and these phosphorylated tyrosine side chains serve as docking sites for cytoplasmic proteins. As a result

of binding, these adapter molecules become phosphorylated and activated, which results in the activation of downstream signaling pathways. According to the classical dogma, receptor dimerization and activation are controlled mainly by the ECD that adopts a closed conformation in the resting state, which is able to bind the ligand but the dimerization arm located in this region is buried. Ligand binding may induce a transition into the open conformation; or according to other theories, the conformation of the receptor may fluctuate between the closed and open states, and ligand may stabilize the latter one. In the resulting open conformation, association of receptor subunits can happen through the exposed dimerization arms.

According to the classical activation model, the transmembrane domain (TMD), in contrast to the primary ECD, plays only a passive role in the process by merely acting as a membrane anchor. However, several lines of evidence suggest that the TMD does have a remarkable role in receptor activation. For example, a mutation in this region (as in the case of NeuT, a mutant variant of ErbB2) can significantly influence the association, thus the activation tendency of the receptors. Consistently, two so-called Sternberg-Gullick dimerization GxxxG motifs have been described in the TMD of ErbB proteins, which can mediate the dimerization of TMDs. The membrane helices of the members of this receptor family contain two such sequences (except for ErbB3) that can mediate the formation of two functionally distinct dimers. The C-terminal motif mediates the formation of functionally inactive dimers, while the ones formed through N-terminal GxxxG motifs are active, as the latter type of association induces a reorientation of kinase domains leading to their activation, which can activate downstream signaling cascades. The two transmembrane conformations can be easily converted into each other through a rotational movement. Based on novel observations, the activation of ErbB proteins can be modeled by a rotational activation model that suggests that the TMD and the kinase domains have intrinsic dimerization and self-activation tendency, which is counteracted by the tethered conformation of the extracellular domain, by the formation of inactive kinase dimers and by interactions of the juxtamembrane segment with anionic lipids of the membrane. This inhibitory mechanism is relieved by ligand binding to the extracellular domain, thus inducing a reorientation in the transmembrane and intracellular domain, resulting in the activation of the kinase domain and induction of signaling pathways. The magnitude of the dipole potential might influence the dimerization and activity of ErbB proteins through effects on their TMD.

### 1.3. Lipid rafts

Another level of complexity of the cell membrane originates from the inhomogeneous lateral distribution of its lipids, which leads to the formation of membrane microdomains of submicron or nanometer scales, termed lipid rafts, with special lipid and protein composition, and supramolecular architecture. According to the classical lipid raft theory, the lateral association of sphingolipid and cholesterol molecules leads to the formation of these microdomains that are characterized by tight packing density, highly resembling the liquid ordered domains of model membranes in many aspects. Membrane proteins are segregated into or out of these areas according to their affinity for raft and non-raft lipid phases, and this distribution can significantly influence their functions through changing the probability of their interactions, and modifying their conformations. According to the initial consensus definition of these microdomains, “membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes”. According to this definition, the underlying mechanism of this phase separation is based on liquid–liquid immiscibility of certain lipid species, and proteins do not have an active contribution to the process. Lipid–protein assemblies arising from alternative phenomena would thus not qualify as rafts. However, the raft concept has been controversial from the initial description of the hypothesis, leading to continuous refinement of the definition. As of the most widely accepted recent views, the formation of rafts is actively influenced by the proteins residing in these microdomains, the cortical actin network according to the “picketed fence” model, and certain intracellular organelles (like the endoplasmic reticulum) through direct contacts with the membrane.

Selective labeling of lipid rafts is possible by using certain molecules due to their preferential concentration in these microdomains. Although none of these markers can be considered ideal, the most widely used and accepted methods include transfecting cells with plasmids encoding glycosylphosphatidylinositol (GPI)-anchored proteins, or labeling them with subunit B of cholera toxin (CTX-B) that selectively binds to GM1 ganglioside or antibodies (for example AC8) recognizing ordered arrays of cholesterol molecules characteristic for rafts.

Lipid rafts play important roles in a great variety of cellular processes including signaling, membrane trafficking, differentiation, adhesion or migration of cells, synaptic transmission, cytoskeletal organization or cellular entry of pathogens. Besides these effects, rafts can influence the function of ErbB proteins, as for example, the raft resident GM3 ganglioside was shown to inhibit the activation of EGFR.

Since lipid rafts are characterized by a special composition distinct from that of other regions of the membrane, and the magnitude of the dipole potential is mainly determined by the composition of the membrane, it seems logical to assume that the magnitude of dipole potential may be different in the raft and non-raft membrane microdomains. Although atomic-force microscopic measurements in model membranes suggested that the dipole potential is larger in liquid-ordered domains, and heterogeneity observed in the fluorescence signals of di-8-ANEPPS in living cells has been assumed to be caused by lipid rafts, explicit proof and quantitative analysis of this correlation has not yet been presented.

#### **1.4. Gaucher's disease**

Alterations in the lipid composition of the membrane are associated with certain diseases. One such pathological condition characterized by a changed membrane composition is the most frequent lysosomal storage disorder, Gaucher's disease in which glucosylceramide accumulates because of a deficiency of the glucocerebrosidase enzyme. Besides the neurological and musculoskeletal abnormalities and visceral symptoms (as hepato- and splenomegaly), the most pathognomic alteration is the presence of the so-called Gaucher cells that are abnormally activated macrophages resulting from the accumulated glycosylceramide.

Due to the relatively low frequency of the disease and the difficulties in obtaining samples from patients, different model systems are commonly used in the examination of the pathomechanism of Gaucher's disease. One of the most widely used tools is the application of monocytes differentiated into macrophages using phorbol-12-myristate-13-acetate (PMA). If conduritol B epoxide (CBE) is used simultaneously with PMA during the differentiation, macrophages develop a phenotype characteristic of Gaucher's disease as a result of glucocerebrosidase inhibition.

The changed lipid composition of the cell membrane due to enzyme deficiency has been demonstrated both in samples obtained from Gaucher patients and in model systems of the disease. Alterations in membrane composition include significantly elevated levels of ceramides, di- and trihexosylceramides, sphingomyelin and phosphatidylglycerol, not only in the lysosomes, but also in other endosomal compartments and the cell membrane, particularly in lipid rafts. These modified membrane compositions were suggested to play roles in the process of abnormal macrophage activation as well.

## 2. Objectives

The key event of the activation of ErbB receptors is the homo- or heterodimerization of the proteins. Although the extracellular domain was initially suggested to be primarily important in the process of association, based on recent results, the transmembrane domain provides an active contribution as well. Since the TMD is located in the hydrophobic interior of the membrane, i.e. in the region of the dipole potential, changes in its magnitude may influence the conformation and association of these domains, thus also the activation of the receptors and the resulting initiation of signaling pathways. However, the importance of the dipole potential in the process has not been tested yet, so we set out to:

- a) determine the effects of treatments (phloretin and 6-ketocholestanol) previously published to modify the dipole potential in our experimental system,
- b) examine the effects of changing the dipole potential on the cell surface expression of EGFR and its ligand affinity,
- c) analyze the alterations in the association processes of EGFR, ErbB2 and NeuT proteins in response to changes in the dipole potential,
- d) test the functional consequence of the treatments above,
- e) examine if the effects depend on the presence of lipid rafts.

Since the magnitude of the dipole potential is primarily determined by the lipid composition of the membrane, it is reasonable to assume that the dipole potential is larger in the lipid rafts possessing a special composition than in the bulk phase of the membrane. However, this relationship has not been demonstrated directly in living cell membranes. It seems also logical that the magnitude of the dipole potential can be altered in diseases characterized by a changed membrane composition. Thus, in the second phase we examined:

- a) the spectral changes of new voltage sensitive 3-hydroxyflavone derivatives (F66 and PPZ8) in response to dipole potential modifying treatments,
- b) the correlations between the dipole potential-dependent intensity ratios of various voltage sensitive fluorophores and the intensities of raft and non-raft membrane domain markers,
- c) the magnitude of differences in the the dipole potential between different membrane microdomains,
- d) the alterations in the emission spectra of F66 and PPZ8 localized in rafts vs the bulk phase of the membrane,
- e) and the changes in the magnitude of the dipole potential in a model system of Gaucher's disease, a disorder characterized by alterations in the lipid composition of the cell membrane (and lipid rafts, in particular).

### **3. Materials and methods**

#### **3.1. Cell lines, treatments and transfection of cells**

In our experiments, we used human breast carcinoma-derived SKBR-3 cells expressing high amounts of ErbB1 and ErbB2, human epithelial carcinoma-derived A431 and cervix carcinoma-derived HeLa cells with high and medium levels of EGFR, respectively, Chinese hamster ovary (CHO) cells without ErbB expression and a modified CHO-derived cell line, F1-4, that stably expresses the ErbB1-eGFP fusion protein. For experiments involving growth factor stimulation serum-starved cells were incubated with 100 ng/ml (~16 nM) of recombinant human EGF for 5 min at 37 °C. In certain cases, the dipole potential was increased and decreased by treating cells with 6-ketocholestanol and phloretin, respectively at a concentration of 100 μM for 10 min at room temperature in the presence Pluronic F-127.

For transient transfection, cells were lipofected using Lipofectamine 2000 or electroporated with Amaxa Nucleofector. In our intracellular phosphorylation measurements, cells were transfected with ErbB2-pcDNA3 or pSV2neuNT plasmid coding for the wild-type ErbB2 or the human NeuT protein, respectively, while for number&brightness experiments PCDNA3.1 ErbB2-short-mYFP and PCDNA3.1 ErbB2 (Val659Glu)-short-mYFP plasmids were used. Plasmids encoding GFP-GPI were utilized for lipid raft labeling.

As a model of Gaucher's disease, acute monocytic leukemia derived THP-1 cells were differentiated into macrophages by PMA treatment (50 ng/ml for 5 days) and the Gaucher phenotype was induced by a simultaneous treatment with 500 μM CBE.

#### **3.2. Labeling of the cells with dipole potential sensitive fluorophores, raft and non-raft markers**

Di-8-ANEPPS, F66 and PPZ8 fluorophores were applied to determine the magnitude of the dipole potential using confocal microscopy. Cells grown on 8-well chambered coverglass were labeled with 2 μM di-8-ANEPPS for 10 minutes at 12°C or with 10 nM F66 or PPZ8 for 20 minutes on ice.

For the visualization of lipid rafts in the membrane, cells were transfected with plasmids encoding GFP-GPI, labeled with 8 μg/ml AlexaFluor647-conjugated subunit B of cholera toxin or 10 μg/ml AC8 anti-cholesterol antibody for 20 minutes on ice. The latter antibody was visualized with Cy5-conjugated GAMIG Fab fragments. Labeling of non-raft membrane domains was carried out using 25 μg/ml AlexaFluor647-conjugated transferrin.

### **3.3. Determination of the effects of the dipole potential on EGF binding**

In order to determine the effects of the dipole potential on the EGF binding affinity of ErbB1, we measured the binding of tetramethylrhodamine-conjugated EGF (TAMRA-EGF) to the receptor using flow cytometry after modifying the dipole potential of starved SKBR-3, A431 or HeLa cells (non-competitive method). Cells were incubated with a dilution series of labeled EGF for 30 minutes on ice. Alternatively, the binding of TAMRA-EGF to ErbB1 was determined in the presence of different concentrations of unlabeled EGF (competitive method). Mean fluorescence intensities were determined in each sample and plotted as a function of the applied concentration of labeled or unlabeled EGF in the non-competitive and competitive methods, respectively. The appropriate Hill equation was fitted to the binding data, and parameters characterizing the EGF binding affinity were calculated.

### **3.4. Analysis of the effects of dipole potential on the association of ErbB proteins**

First, flow cytometric Förster resonance energy transfer (FRET) measurements were carried out to determine the effects of changing the dipole potential on ErbB receptor dimerization. Untransfected SKBR-3 cells were used in the analysis of ErbB2 homoassociation and ErbB1-ErbB2 heteroassociation, ErbB1 homoassociation was measured in untransfected HeLa cells, while CHO cells were electroporated with ErbB2-pcDNA3 or pSV2neuNT plasmids to examine the associations of wild-type ErbB2 or NeuT, respectively. NeuT carries a Val→Glu mutation in the transmembrane domain. The dipole potential of starved cells was changed and some of the samples were stimulated with EGF followed by labeling the cells with AlexaFluor546-conjugated (donor) or AlexaFluor647-conjugated (acceptor) anti-ErbB1 Ab11 or anti-ErbB2 trastuzumab antibodies for 30 minutes on ice. Fluorescence intensities corresponding to donor, FRET and acceptor channels were determined on a cell-by-cell basis and mean FRET efficiencies characterizing the extent of association were calculated after applying the appropriate correction factors.

As an alternative method to FRET measurements, the associations of the receptors were quantified with number&brightness (N&B) analysis of confocal images. F1-4 cells stably expressing ErbB1-eGFP fusion proteins were used to determine EGFR homoassociation, while HeLa cells were transfected with PCDNA3.1 ErbB2-short-mYFP and PCDNA3.1 ErbB2 (Val659Glu)-short-mYFP plasmids for the investigation of ErbB2 and NeuT, respectively. The dipole potential of starved cells grown on 8-well chambered coverglass was modified followed by EGF stimulation of certain samples. Confocal image series were recorded of the cell membrane region adjacent to the coverslip and the mean and the variance of the fluorescence

intensity were determined in every membrane pixel. Finally, the average molecular brightness value characterizing the clustered state of the molecule was calculated from pixelwise data.

### **3.5. Examination of the functional significance of changing the dipole potential and its dependence on the presence of lipid rafts**

Control starved SKBR-3 cells and those with an altered dipole potential were treated with EGF, fixed and permeabilized, which was followed by labeling the cells for 30 minutes on ice with antibodies, at a concentration of 10  $\mu\text{g/ml}$ , against phosphotyrosine (PY99), ErbB2 phosphorylated at Tyr1248 (Ab18) and ErbB1 phosphorylated at Tyr1068 (1H12). Primary antibodies were visualized by secondary staining with 20  $\mu\text{g/ml}$  AlexaFluor647-conjugated goat-anti-mouse antibodies (GAMIG) for 30 minutes on ice. The mean fluorescence intensities of individual cells were determined using flow cytometry. We repeated our measurements on cells electroporated with ErbB2-pcDNA3 or pSV2neuNT plasmids.

In order to examine the relationship between ErbB2 phosphorylation and the presence of lipid rafts, control starved SKBR-3 cells grown on 8-well chambered coverglass and those with an altered dipole potential were treated with EGF and labeled with AlexaFluor546-conjugated trastuzumab and AlexaFluor647-conjugated CTX-B for 30 minutes on ice. After fixation and permeabilization of these cells, Ab18 antibody against phosphorylated Tyr1248 of ErbB2 was applied, which was visualized with AlexaFluor488-GAMIG. Images were taken of the region of the cells adjacent to the coverslip using a confocal microscope. During the evaluation of images, membrane pixels were identified manually and segmented into two masks containing pixels with high or low CTX-B intensity (“CTX-high” and “CTX-low” regions, respectively). The intensity of Ab18 staining (corresponding to tyrosine phosphorylated ErbB2) normalized to the expression level of ErbB2 (trastuzumab staining) was separately calculated for the CTX-high and CTX-low masks corresponding to raft and non-raft domains, respectively. The Pearson correlation coefficients were also calculated between the Ab18 intensity and CTX staining and between trastuzumab intensity and CTX staining.

### **3.6. Determination of the correlation coefficient between the magnitude of the dipole potential and the intensity of membrane microdomain markers**

In order to determine the correlation between the magnitude of the dipole potential and the intensity of membrane microdomain markers, SKBR-3 and A431 cells grown on 8-well chambered coverglass were labeled with dipole potential sensitive fluorophores (di-8-ANEPPS, F66 or PPZ8) and raft (GFP-GPI, AlexaFluor647-CTX-B or AC18 and AlexaFluor647-GAMIG-Fab) or non-raft (AlexaFluor647-transferrin) markers, as described previously. Images were acquired from the flat, bottom membrane of cells adjacent to the coverslip using a confocal laser scanning microscope. During the evaluation of images, membrane pixels were identified manually and the dipole potential sensitive intensity ratios were calculated in these pixels. Finally, the Pearson correlation coefficients between these intensity ratios and the fluorescence intensities of raft or non-raft markers were determined from pixelwise data of individual cells. The confidence intervals of the correlation coefficients assuming no correlation between the analyzed parameters were also determined for each image according to the method of Costes. The correlation coefficient between two lipid raft labels, GFP-GPI and CTX-B, was used as a positive control. Alternatively, evaluation of the images was carried out by excluding the brightest patches with the largest intensities within the cell mask.

### **3.7. Determination of the magnitude of the dipole potential in raft versus non-raft membrane regions**

In order to quantify the differences in the magnitude of the dipole potential between raft and non-raft membrane regions, images taken previously from the flat, bottom membrane of SKBR-3 and A431 cells adjacent to the coverslip were evaluated using a different approach. During image analysis, cell membrane pixels were identified manually and segmented into two masks by determining a threshold intensity for lipid raft labels (CTX-B or GFP-GPI), confirmed by visual inspection. Pixels were considered “raft” or “nonraft” pixels if their intensity was above or below the determined threshold, respectively. Finally, average dipole potential sensitive di-8-ANEPPS and F66 intensity ratios were separately determined from individual pixels in the “raft” and “nonraft” regions. Alternatively, using the lambda mode of the confocal laser scanning microscope, the emission spectrum of F66 or PPZ8 was determined separately inside and outside lipid rafts in a manner identical to what was described for the intensity ratio.

### **3.8. Determination of the magnitude of the dipole potential in control versus Gaucher-type THP-1-derived macrophages**

THP-1 monocytes were differentiated into macrophages by PMA treatment. As a model system of the disease, CBE was applied simultaneously with the differentiation to induce the Gaucher phenotype. The average dipole potential was determined in control and Gaucher-type macrophages using di-8-ANEPPS, F66, or PPZ8 labeling, followed by acquiring images from the midplane of the cells. During processing, segmentation of images into membrane and non-membrane pixels was carried out and the average fluorescence intensity ratio of the cell membrane pixels, characterizing the dipole potential, was calculated after background subtraction.

## **4. Results**

### **4.1. The effects of 6-ketocholestanol and phloretin on the magnitude of the dipole potential**

Although the dipole potential has been shown to influence the structure and function of transmembrane proteins, ErbB receptors have not been examined in this aspect. Thus, in the first phase of our experiments we examined whether changes in the magnitude of the dipole potential can modify the function of ErbB proteins, namely EGFR and ErbB2. According to previous studies, the dipole potential of living cell membranes can be increased and decreased with 6-ketocholestanol and phloretin, respectively. First, we tested the applicability of these compounds by measuring the magnitude of the dipole potential with a confocal microscopic method using an excitation ratiometric approach with the voltage sensitive fluorophore di-8-ANEPPS. After dipole potential modifying treatments, SKBR-3, A431 and HeLa cells were labeled with di-8-ANEPPS and images were taken from the midplane of the cells with a confocal laser scanning microscope. During image processing, mean fluorescence intensity ratios of membrane pixels were determined after excitation at two different wavelengths. Consistent with our expectations, 6-ketocholestanol significantly increased, whereas phloretin decreased the excitation intensity ratio proportional to the magnitude of the dipole potential of the plasma membrane in all cell types used. Based on these measurements, 6-ketocholestanol and phloretin were shown to be useful in changing the dipole potential, so we proceeded to investigate the effect of these alterations on the clustering and signaling properties of ErbB plasma membrane receptors.

### **4.2. The effect of changing the dipole potential on the ligand binding affinity of EGFR**

As ligand binding is the first step of the functional activation of EGFR, we examined if changing the dipole potential results in alteration of ligand binding properties of ErbB1. After modifying the magnitude of dipole potential in SKBR-3, A431 and HeLa cells, the dissociation constant characterizing the binding affinity of EGF to ErbB1 was determined by competitive and non-competitive binding assays using fluorescently labeled EGF. First, in non-competitive experiments, after labeling cells with a dilution series of TAMRA-EGF, mean cellular intensities of the samples were measured using flow cytometry. Dissociation constants were determined by analyzing the binding curves fitted to the Hill equation using the obtained data points. According to our measurements, increasing the dipole potential significantly decreased the EGF binding affinity of ErbB1.

Since incorporation of a fluorescent dye may alter the interaction of EGF with the dipole potential, competitive binding experiments were carried out to exclude the possibility that the

observed inhibited binding of EGF at a high dipole potential was due to the presence of the fluorescent label. Competitive binding curves were measured as described above, but the dilution series contained unlabeled EGF mixed with a constant concentration of labeled EGF. Based on our results, the binding affinity of unlabeled EGF was reduced by a high dipole potential to a similar extent as for fluorescent EGF.

Next, the binding of fluorescent EGF was tested under conditions used for further stimulation experiments. After altering the dipole potential, cells were incubated with 100 ng/ml TAMRA-EGF and mean cellular intensities of the samples were measured using flow cytometry. In order to test the dependence of the observed effect on temperature, experiments were carried out either on ice or at 37 °C. The binding of fluorescent EGF was reduced by ~50% at an elevated dipole potential in the case of both conditions. These results were in good agreement with predictions based on the previously determined binding curves.

Since decreased EGF binding can be theoretically caused by lower cell surface expression of ErbB1, we determined the amount of the receptor in the cell membrane after phloretin and 6-ketocholestanol treatments. After incubation with these compounds, cells were labeled with AlexaFluor546-conjugated anti-ErbB1 Ab11 monoclonal antibodies and the mean cellular fluorescence intensities were measured in each sample. Although EGF binding was significantly modified by the dipole potential, ErbB1 expression was not changed as shown by the unaltered binding of Ab11, which convincingly showed that the ligand binding ability of the receptor was significantly inhibited by an elevated dipole potential.

### **4.3. The effect of the dipole potential on the clustering of ErbB1 and ErbB2**

Since transmembrane signaling induced by EGF is preceded by clustering of ErbB receptors, we investigated whether alterations in the dipole potential have any effect on receptor oligomerization in quiescent or stimulated cells. In these experiments, the levels of associations were determined by two methods: flow cytometric FRET measurements and number&brightness (N&B) analysis using confocal microscopy.

According to both FRET and N&B experiments, the dipole potential exerted only minor effects on the homoassociation of ErbB1 and ErbB2 in non-stimulated cells. ErbB1 was mainly monomeric in unstimulated cells, since the molecular brightness of ErbB1-EGFP (0.07) was comparable to that of monomeric EGFP (0.06). On the other hand, ErbB2 formed clusters even in quiescent cells judged from the almost threefold difference between the molecular brightness of ErbB2-mYFP (0.08) and that of monomeric mYFP (0.032). Homoclustering of the TMD mutant NeuT was significantly enhanced even in unstimulated cells by an elevated dipole

potential according to both FRET and N&B measurements. Although the homoclustering of transfected wild-type ErbB2 was also increased by the dipole potential according to FRET measurements, this effect was less significant than that on NeuT (15% and 52% 6-ketocholestanol-induced relative increase in the FRET efficiency for wild-type ErbB2 and NeuT, respectively).

Contrary to resting cells, an elevated dipole potential significantly and systematically promoted the EGF-induced homoassociation of ErbB1 and ErbB2 according to both FRET and N&B measurements. Although a decrease in the dipole potential had a less predictable effect, EGF-induced homoclustering of ErbB2 was significantly inhibited by phloretin.

An elevated dipole potential exerted an approximately 2-times larger effect on the EGF-induced homoclustering of NeuT as compared to that on wild-type ErbB2 according to FRET and N&B measurements. The relative, 6-ketocholestanol-induced change in the FRET values in EGF-stimulated samples were 19% and 40% for wild-type ErbB2 and NeuT, respectively, and the 6-ketocholestanol-induced rise in the molecular brightness values were 13% and 33% under the same two conditions.

Since ErbB2 is the preferred heterodimerization partner of EGF-activated ErbB1, the investigation of the effect of the dipole potential on clusters of ErbB1 and ErbB2 would be incomplete without measuring their heteroassociation.

According to flow cytometric FRET measurements, the dipole potential did not have any effect on the heteroclustering of ErbB1 with ErbB2 in quiescent cells, while the EGF-induced increase in the interaction of the two receptors was significantly higher in cells in which the dipole potential was elevated. Based on these results we concluded that the homo- and heteroassociations of ErbB1 and ErbB2, especially in ligand-stimulated cells, show a positive correlation with the dipole potential.

#### **4.4. The effect of changing the dipole potential on EGF induced signaling**

Ligand-induced clustering of ErbB proteins leads to phosphorylation of tyrosine residues in the receptors and spreading of the tyrosine phosphorylation signal. In order to test whether the dipole potential-related changes observed in the clustering of ErbB1 and ErbB2 are mirrored in their signaling, the level of tyrosine phosphorylation was determined in quiescent and EGF-stimulated cells using antibodies against phosphotyrosine (PY99), ErbB1 phosphorylated at Tyr1068 (1H12) and ErbB2 phosphorylated at Tyr1248 (Ab18). For these measurements, after modification of the dipole potential and EGF stimulation of certain samples, SKBR-3 cells were labeled with the previously described primary antibodies that were visualized with Alexa647-

conjugated GAMIG secondary antibodies followed by determination of mean cellular fluorescence intensities in each sample using flow cytometry. We repeated our experiments using HeLa cells transfected without any plasmid, or with ErbB2-pcDNA3 or pSV2neuNT plasmids encoding wild-type ErbB2 and NeuT proteins, respectively.

Based on our results, alteration of the dipole potential did not modify the low level of tyrosine phosphorylation in unstimulated cells, while the EGF-induced response was correlated with the dipole potential. Similar to the FRET and N&B measurements, the effect of the dipole potential on NeuT was more pronounced than on wild-type ErbB2. We concluded that the growth factor-induced tyrosine phosphorylation response of cells was also significantly modified by the dipole potential.

#### **4.5. Correlation between the activation of ErbB2, its lipid raft localization and the dipole potential**

Since the dipole potential is assumed to be different in lipid rafts than in the rest of the membrane, some sort of correlation can be expected between the activation state of ErbB2, its raft localization and the dipole potential. In order to examine this correlation, control serum-starved cells and those with an increased dipole potential were stimulated with EGF followed by staining with AlexaFluor546-conjugated trastuzumab and AlexaFluor647-conjugated CTX-B. Afterwards, cells were fixed and labeled with Ab18 antibodies against phosphorylated ErbB2, which was visualized by AlexaFluor488-conjugated GAMIG. Confocal images were taken of the cell membrane regions adjacent to the coverslip. During image analysis, cells were manually identified and membrane regions were segmented into two masks corresponding to high and low CTX intensities. The intensity of Ab18 staining (corresponding to tyrosine phosphorylated ErbB2) normalized to the expression level of ErbB2 (trastuzumab staining) was separately calculated for the high-CTX and low-CTX masks corresponding to raft and non-raft domains, respectively. Besides this, the Pearson correlation coefficient was calculated between the Ab18 intensity and CTX staining and between trastuzumab intensity and CTX staining.

In cells with an unmodified dipole potential there was no difference between the normalized tyrosine phosphorylation of ErbB2 inside and outside lipid rafts. Increasing the dipole potential enhanced the activation state of ErbB2 and it did so more pronouncedly outside lipid raft regions. EGF stimulation led to ErbB2 tyrosine phosphorylation both inside and outside lipid rafts in cells with an unmodified dipole potential and the increase was larger in regions outside lipid rafts. EGF stimulation in cells with an increased dipole potential did not increase the normalized tyrosine phosphorylation of ErbB2 further.

Our results suggested that the 6-ketocholestanol-induced increase in the dipole potential elevates ErbB2 tyrosine phosphorylation more significantly outside lipid rafts. The fact that EGF stimulation led to a larger rise in ErbB2 tyrosine phosphorylation outside lipid rafts suggests that the growth factor either preferentially activates ErbB2 outside lipid rafts or activated ErbB2 migrates out of lipid rafts. This assumption was corroborated by a significant decrease in the correlation coefficient between phosphorylated ErbB2 and CTX staining upon EGF and 6-ketocholestanol treatments, while the effects on the correlation between ErbB2 and CTX intensities were less substantial. In conclusion, the results suggest that activation of ErbB2 by either EGF or increased dipole potential preferentially takes place outside lipid rafts.

#### **4.6. The response of emission ratiometric dyes to changes in the dipole potential**

Our observations in the first phase of our work suggested that lipid rafts might play a role in the effects of the dipole potential on ErbB proteins. Although it was hypothesized earlier that due to their special lipid composition the magnitude of the dipole potential is larger in membrane rafts than in the bulk phase of the membrane, this assumption has not been demonstrated directly in living cells. Thus, in the second phase of our experiments, we set out to prove that the dipole potential is indeed stronger in lipid rafts than in non-raft membrane regions. To do so, it was necessary to find methods suitable to demonstrate the difference in the magnitude of the dipole potential between the two regions, and to rule out the possibility that changes in other membrane biophysical parameters (for example fluidity) can result in the observed differences between the two microdomains. Thus, we needed to supplement our measurements applying di-8-ANEPPS in an excitation ratiometric method. Two novel 3-hydroxyflavone derivatives, the PPZ8 and F66 fluorophores appeared to be good candidates for this purpose using emission ratiometric assays, especially when both of them are applied in the same experimental system, since their chromophores are oriented in opposite directions with respect to the bilayer plane. As a result, their spectral changes are complementary to each other upon alterations in the intramembrane electric field. However, the direction of spectral changes due to alterations of another parameter (for example fluidity) are alike in the case of both dyes. Thus, in the first part of the second phase of our experiments, we tested whether two oppositely oriented voltage-sensitive fluorophores, PPZ8 and F66, are suitable for determining changes in the dipole potential in living cell membranes. We treated A431 and SKBR-3 cells with 6-ketocholestanol, and after labeling we recorded images from the flat membrane of the cells adjacent to the coverslip. We measured fluorescence emission in two wavelength ranges, corresponding to the N\* and T\* bands, and the

ratio of the two intensities correlating with the magnitude of the dipole potential was determined on a pixel-by-pixel basis.

Consistent with our expectations, 6-ketocholestanol significantly increased the N\*:T\* intensity ratio of PPZ8, while decreasing it in the case of F66. Both the shift in the pixelwise distribution of the emission ratio and changes in its mean confirmed that PPZ8 and F66 can be used to determine changes in the dipole potential in living cells via an emission ratiometric method using confocal microscopy.

#### **4.7. Correlation of the dipole potential with the distribution of markers for raft and non-raft domains**

In order to test the hypothesis that the largely different lipid composition of rafts leads to a higher dipole potential compared to the bulk phase of the membrane, we determined the correlation coefficients between the dipole potential sensitive ratiometric responses of three dyes (di-8-ANEPPS, PPZ8 and F66) and the intensities of three commonly used lipid raft markers (AlexaFluor647-labeled CTX-B, GFP-GPI and anti-cholesterol AC8 antibody) in the membranes of living A431 and SKBR-3 cells. The chromophore groups of PPZ8 and di-8-ANEPPS are oriented parallel to each other, whereas the fluorophore of F66 is aligned in the opposite direction.

First, as a positive control, we determined the correlation coefficient between two widely accepted raft markers. Cells were transfected with plasmids encoding GFP-GPI and labeled with CTX-B, and images were recorded of the flat, bottom membrane region adjacent to the coverslip. The mean Pearson correlation coefficient between the pixelwise intensities of the two raft markers was 0.55 in A431 and 0.48 in SKBR-3 cells. The fact that these correlation coefficients are far outside the confidence intervals of the correlation coefficient, assuming no correlation, confirms a strong positive correlation and allowed us to conclude that the method can be used to examine the correlation between two signals in living cells.

Then, we determined the relationship between the dipole potential and the intensity of raft markers. Cells were labeled by one of the three dipole potential sensitive fluorophores, the intensity ratios proportional to the dipole potential were determined on a pixel-by-pixel basis, and their correlations with the intensities of the raft markers (CTX-B, GFP-GPI, or anti-cholesterol antibody) were calculated. The excitation ratio of di-8-ANEPPS showed a positive correlation with each of the lipid raft markers. On the contrary, the F66 N\*:T\* emission ratio correlated negatively with the intensity of CTX-B. The statistical significance of these findings is supported by the fact that the calculated correlation coefficients were outside the confidence

intervals determined for the absence of correlation. Their biological significance is underlined by the fact that the correlation coefficients between the dipole potential and the raft markers are similar to what was determined in the case of the positive control. We also investigated the relationship between the dipole potential reported by PPZ8 and the lipid raft marker, CTX-B, and a negative correlation was observed. Because the chromophore in F66 is oriented oppositely to that in di-8-ANEPPS and PPZ8 with respect to the membrane bilayer, the dipole potential correlates positively with the intensity ratio of di-8-ANEPPS and PPZ8 and negatively with the emission ratio of F66. It follows from this relationship that the results obtained with the two oppositely oriented dipole potential-sensitive fluorophores, di-8-ANEPPS and F66, imply that the magnitude of the dipole potential correlates with the presence of raft microdomains, whereas the results obtained with PPZ8 contradict this conclusion, which will be resolved later.

In order to get an independent confirmation of our results, we also investigated the relationship between a non-raft marker, transferrin receptor, and the dipole potential. Consistent with our previous findings, the excitation ratio of di-8-ANEPPS correlated negatively with the distribution of fluorescently labeled transferrin in both SKBR-3 and A431 cells, implying that the dipole potential is indeed weaker in nonraft domains than in rafts.

#### **4.8. The magnitude of the dipole potential in lipid rafts and other membrane regions**

Although the results presented in the previous section convincingly show that the dipole potential correlates positively with the localization of lipid rafts, they do not reveal how much the dipole potential differs in lipid rafts and in the bulk phase of the membrane. We quantified this difference using an alternative approach. Similar to the previous method, cells transfected with plasmids encoding GFP-GPI or labeled with CTX-B were stained with the dipole potential-sensitive dyes di-8-ANEPPS or F66. Images acquired from the bottom of the cells were segmented into “raft” and “non-raft” regions on the basis of their GFP-GPI or CTX-B intensities. The average di-8-ANEPPS or F66 intensity ratios were separately determined for individual pixels in the “raft” (CTX-B high and GFP-GPI high) and “non-raft” regions, and the ratio determined for the raft region was normalized to that calculated for the non-raft region in every cell.

Consistent with our previous results, the di-8-ANEPPS excitation ratio was significantly higher in raft regions than in non-raft regions in both A431 and SKBR-3 cells, whereas an opposite relationship was revealed when determining the emission ratio of F66, which was significantly smaller in rafts. Given the opposite orientation of the fluorophores in the two dipole potential sensitive dyes, both of these findings imply that the dipole potential is higher in rafts

than in the rest of the membrane. These observations confirmed the hypothesis that the magnitude of the dipole potential is larger in raft microdomains than in other regions of the living cell membrane.

#### **4.9. The emission spectra of 3-hydroxyflavone dyes in lipid rafts and other membrane regions**

We also measured the emission spectra of the 3-hydroxyflavone dyes inside and outside lipid rafts to estimate the magnitude of the spectral changes between different membrane regions. After labeling the cells as described previously, the emission spectrum was measured for each individual pixel, and they were separately averaged for pixels in the raft and non-raft regions. The most notable difference between the two spectra was the significantly lower intensity in the non-raft regions compared with raft pixels, possibly because of water-induced quenching of fluorescence outside rafts. Another important difference was that although both the N\* and T\* species contribute significantly to the emission of F66 in the 460–520 and 550–600 nm regions, respectively, PPZ8 exhibits practically no peak in the spectral region corresponding to the N\* species. This phenomenon can explain the inability of PPZ8 to faithfully report the dipole potential difference inside and outside lipid rafts.

Then, we determined the emission ratio of F66 inside and outside rafts by integrating the emission spectrum in the spectral range corresponding to the N\* and T\* species both in the presence and absence of 6-ketocholestanol treatment. The results implied that 6-ketocholestanol changes the emission ratio of F66 approximately to the same extent inside and outside rafts, suggesting that it increases the dipole potential similarly in both membrane microdomains.

#### **4.10. Changes in the dipole potential in response to sphingolipid accumulation characteristic of Gaucher's disease**

Because the magnitude of the membrane dipole potential is mainly determined by the lipid composition, it can be altered in diseases in which the composition of the cell membrane changes. Since Gaucher's disease has been shown to lead to significant alterations in the lipid content of cell membranes in general and lipid rafts in particular, we tested whether these changes are substantial enough to alter the dipole potential in an *in vitro* model of Gaucher disease. Control and Gaucher-type, THP1-derived macrophages were labeled with the voltage sensitive di-8-ANEPPS, PPZ8, or F66 dyes followed by acquiring confocal microscopic images in which the fluorescence intensity ratio, characteristic of the dipole potential, was evaluated in the identified membrane pixels only.

CBE treatment, inducing the Gaucher phenotype, led to a significant increase in the excitation ratio of di-8-ANEPPS and in the emission ratio of PPZ8, while causing a significant decrease in the emission ratio of F66. Results obtained with the three different dipole potential sensitive fluorophores strongly support that sphingolipid accumulation, characteristic of Gaucher's disease, significantly increases the magnitude of dipole potential in the cell membrane. Our findings suggest that relevant changes in the raft-like lipid composition due to pathological conditions can change the dipole potential in living cells, which might possibly play a role in the pathomechanism of these diseases.

## **5. Discussion**

### **5.1. The relationship between lipid rafts and ErbB proteins**

Although creating the largest electric field, the dipole potential is the least known of the three described membrane potentials. Despite being shown to modify the structure and function of transmembrane proteins by influencing the stability and conformation changes of their TMD, the effects of the dipole potential have not been examined on the function of receptor tyrosine kinases. Since the TMD was suggested to play an active role in the action of ErbB proteins, the assumption seems indeed logical that the dipole potential might influence the function of these receptors.

Thus, in the first part of our experiments, we examined the effects of the dipole potential on the function of ErbB proteins, and by using flow cytometric FRET measurements and N&B analysis of confocal microscopic images we showed that the dipole potential is significantly correlated with the homo- and heteroclustering of ErbB1 and ErbB2. The effect was systematically observed in EGF-stimulated cells, whereas changes in the dipole potential did not exert substantial effects on ErbB1 and ErbB2 in unstimulated cells. The increased growth factor-induced association of ErbB proteins at an elevated dipole potential was correlated with enhanced signaling, as confirmed with measurements of receptor phosphorylation levels. However, the fact that increased signaling at an elevated dipole potential was associated with reduction of ligand binding was unexpected. If this suppressed ligand binding at an elevated dipole potential is taken into account, the dipole potential-induced augmentation of the EGF effect is even larger since a lower amount of bound ligand brings about the larger changes in the clustering and phosphorylation of ErbB1 and ErbB2. This apparent contradiction can be resolved by assuming that the increase of the dipole potential favors the active conformation of the transmembrane domain dimers, which eventually induces conformational transitions in the extra- and intracellular domains as well resembling or identical to those induced by growth factor binding.

Lipid rafts are important in EGF-mediated signaling since both ErbB1 and ErbB2 have been shown to be raft resident. These microdomains are presumed to play a bipartite role in the clustering and signaling of ErbB proteins, since they inhibit ligand binding but potentiate growth factor-induced signaling at the same time. Our results show that the effects of the dipole potential exactly mirror the bipartite effect of lipid rafts. The dipole potential-induced activation of ErbB2 was more pronounced outside lipid rafts, while the magnitude of increase in the dipole potential induced by 6-ketocholestanol treatment is similar in different membrane microdomains. This might be caused by the fact that the magnitude of the dipole potential might be high enough in

rafts to stabilize the conformation of the TMD permissive for dimerization, even in the absence of 6-ketocholestanol. However, the weaker dipole potential in non-raft microdomains might be unable to stabilize the conformation of TMD required for dimerization. Thus, increasing the dipole potential can exert a substantial effect on dimerization and activation.

We considered six possible models for explaining the phenomenon that the dipole potential only affects ligand-induced clusters without influencing constitutive ones.

1. The dipole potential may only exert a permissive effect on clustering, which is not strong enough for altering the weak and transient constitutive dimers or oligomers.
2. It has been suggested that constitutive dimers or oligomers are generated by corralling or confinement by the cytoskeleton, while actively signaling receptor clusters are held together by protein-protein interactions. Since it is unlikely that the membrane dipole potential affects corralling and confinement, it only exerts effects on ligand-induced clusters.
3. The interactions between the electric fields generated by the atoms play important roles in the stabilization of the  $\alpha$ -helical structure of TMDs because of the large dipole moments of peptide bonds. TMDs can actively influence the association, thus the activation of membrane proteins via their specific dimerization motifs, for example the Sternberg-Gullick GxxxG consensus sequence. The TMDs of ErbB proteins (except for ErbB3) contain two such GxxxG motifs that mediate the formation of two distinct dimers with different conformations. These two can easily transition into each other by a simple sliding movement and a rotation of 120 degrees. The GxxxG sequence closer to the C-terminal of the helix mediates the formation of a functionally inactive dimer, while dimers associated through the N-terminal motif are functionally active. According the most widely accepted rotational activation model, the equilibrium between the two functionally different dimers is shifted towards active dimerization in response to ligand binding, which leads to the activation of intracellular domains of receptor subunits. Our experimental results completely fit into this theory. The effects of dipole potential on receptor association can be explained by the fact that parallel  $\alpha$ -helices, present in dimers of single-pass transmembrane proteins, exert a repulsive electrostatic force on each other. According to our semiquantitative model, the magnitude of the electric potential difference behind this effect is comparable to that of the membrane dipole potential. Increasing the dipole potential may counteract the repulsion between the N-termini of helices, which would lead to the stabilization of the interaction between the N-terminal dimerization motifs. On the other hand, the dipole potential does not exert such an effect on the C-terminal motifs oriented parallel with the dipole potential. Thus, the equilibrium between the two forms can be shifted towards formation of active dimers in

response to an increase in dipole potential. Since the importance of such interhelical interactions between TMDs has been described in a great variety of membrane proteins (for example integrins, cytokine receptors, other receptor tyrosine kinases), our theory could be applied to these proteins as well. Assuming that our hypothesis is correct, the magnitude of the dipole potential might influence the association processes of many biologically relevant molecules in the membrane of living cells.

4. Exposure of the TMD helix termini to water decreases their dipole moment and the resulting repulsion between them due to a phenomenon called “solvent screening”. Consequently, if the increase of dipole potential is associated with decreased membrane thickness, less repulsion between TMDs would favor receptor clustering, thus explaining our results. However, it has been shown that neither phloretin, nor 6-ketocholestanol changes the membrane thickness arguing against this model.
5. Consistent with the rotational activation model, the role of the juxtamembrane domain (JMD) has been demonstrated in the dimerization and the resulting activation of ErbB proteins. The antiparallel dimer formation between the N-terminal  $\alpha$ -helical juxtamembrane (JM-A) segments directly connected to TMD dimers is necessary for the asymmetrical dimer formation of the kinase domains, which is required for their activation. However, interaction between the positively charged lysine and arginine amino acids of the JM-A segments and kinase domains and the anionic lipids in the inner leaflet of the cell membrane results in anchoring these regions to the membrane, which leads to the inactivation of kinase domains by making the formation of JM-A antiparallel dimers impossible, thus inhibiting activation of the receptors. TMDs are also important in formation of the active conformation, since the C-termini of transmembrane helices move apart because of the association via the N-terminal GxxxG motifs, which results in the dissociation of JM-A segments from the membrane. The presence of ECD inhibits TMD dimer formation mediated by the N-terminal motifs, since it keeps away the N-termini of transmembrane helices from each other. Ligand binding results in a conformational change of the ECD, which in turn releases the inhibition of TMDs, thus inducing the formation of active TMD dimers and the activation of the kinase domains.

The dipole potential can modify this process by promoting the interaction between the N-terminal dimerization motifs of TMDs, as described previously, and by directly influencing the conformation of JMDs. Since the JMD is embedded in the hydrophobic core of the membrane, the positively charged lysine residues in the JM-A segment may be repelled by the positive lobe of the dipole potential, thus decreasing the stability of the inhibited conformation. In this way, an increased dipole potential may destabilize the membrane

embedding of the JM-A segment favoring the formation of JMD dimers and the activation of kinase domains.

6. Changes in the dipole potential and consequent alterations in lipid-protein interactions may change the distribution of ErbB receptors between raft and non-raft domains. The majority of studies found that lipid rafts inhibit ligand binding and signaling mediated by ErbB1 and that activation takes place upon concomitant migration of ErbB1 out of lipid rafts. Our results imply that activation of ErbB2 by EGF preferentially takes place outside rafts and an increased dipole potential mimics this effect. Therefore, dipole potential-induced redistribution of receptors can also enhance their association and signaling.

By one or more of these mechanisms, ErbB monomers become prone to dimerization after an elevation in the dipole potential. In this way, more monomers are available to form homo- and heterodimers after growth factor stimulation. Therefore, EGF-induced formation of ErbB1-2 heterodimers does not lead to decreased homoassociation of ErbB1 or ErbB2 because the dimers are formed from the dimerization-prone pool of monomers.

The dipole potential may also have implications for the evolution and treatment of cancer. Tumor cells are characterized by an increased raft density and the higher dipole potential in these membrane regions may activate receptor tyrosine kinases in cancer cells. This implies that attenuation of signaling upon lowering the dipole potential may even have therapeutic implications. Our observation emphasize that effects of the dipole potential must not be ignored when examining the effects of membrane environment on receptor clustering.

## **5.2. The relationship between the dipole potential and lipid rafts**

Because cholesterol, membrane compactness, and lipid order have been shown to affect the dipole potential, it was reasonable to assume that the dipole potential is different in liquid-ordered, raft-like membrane microdomains than in the bulk membrane. Although observations supporting this hypothesis have been described in model membranes, and using indirect methods in living cells as well, the correlation has not been demonstrated directly and quantitatively in the membrane of living cells. Therefore, the major aims of the second phase of our work were a) to present evidence for the difference in the dipole potential inside and outside lipid rafts; b) to estimate the magnitude of this difference; and c) to show how large an effect the altered lipid composition of the plasma membrane in metabolic disorders (e.g., Gaucher disease) has on the dipole potential.

Using quantitative evaluation of the Pearson correlation coefficients between the intensities of three different lipid raft markers (GFP-GPI, fluorescently labeled subunit B of

cholera toxin and AC8 antibody against ordered cholesterol array) and the dipole potential sensitive intensity ratios of voltage sensitive dyes (di-8-ANEPPS and F66), we showed that the dipole potential is positively correlated with the presence of lipid rafts. An independent confirmation of the difference in the magnitude of the dipole potential inside and outside rafts was provided by staining non-raft domains with fluorescently labeled transferrin, showing that the dipole potential is significantly weaker in transferrin-labeled nonraft domains than in lipid rafts. The significance of these correlations was evaluated in two ways. The methodological or technical significance was corroborated by the fact that the correlation coefficients were always outside the 95% confidence intervals calculated for the absence of correlation. To appreciate the biological significance of the finding, as a positive control, we determined the correlation coefficient between two widely accepted lipid raft markers (GFP-GPI and CTX-B). Because the correlation coefficients between the dipole potential and lipid raft markers were similar in magnitude to the positive control, we conclude that the observed correlation is strong and biologically meaningful.

Evaluation of the dipole potential sensitive intensity ratios inside and outside lipid rafts enabled us to estimate the magnitude of the lipid raft-dependent increase in the dipole potential. Because a 15% change in the excitation ratio of di-8-ANEPPS represents a 100 mV change in the dipole potential, a difference of ~7% in the excitation ratio of di-8-ANEPPS measured inside and outside lipid rafts corresponds to a ~50 mV alteration in the dipole potential. An independent way of estimating the magnitude of the raft-dependent increase in the dipole potential is to compare the changes in the emission ratio of F66 induced by 6-ketocholestanol and by the presence of lipid rafts. Therefore, we analyzed the dipole potential and its 6-ketocholestanol-induced alteration inside and outside lipid rafts by using a more reliable indicator, F66. 6-ketocholestanol decreased the emission ratio of F66 by ~35% both inside and outside lipid rafts, implying that its effect on the dipole potential is identical in both domains of the membrane. Based on our previous measurements, 6-ketocholestanol induces a ~25% increase in the excitation ratio of di-8-ANEPPS, corresponding to a ~160-170 mV change in the dipole potential according to the calibration published in the literature. Assuming that the 35% change in the emission ratio of F66, induced by 6-ketocholestanol, corresponds to a 160–170 mV difference in the dipole potential, a difference of ~20% in the emission ratio of F66 observed inside and outside lipid rafts is equivalent to a difference of ~100 mV in the dipole potential. Given the large variation in the calculated or measured values of the dipole potential in the literature, the two estimations outlined above are in reasonable agreement with each other.

Although the correlations among three lipid raft markers, GFP-GPI, CTX-B, and the anti-cholesterol antibody, and two dipole potential sensitive indicators, F66 and di-8-ANEPPS, imply the existence of a stronger dipole potential in lipid rafts, the results obtained with the indicator PPZ8 are in disagreement with those collected with the other indicators. In order to explain this contradiction, the photophysical properties of 3-hydroxyflavone dyes, F66 and PPZ8, must be taken into account. These dyes are present in two different ground-state forms: 1) a hydrogen-bonded form (hydrated, H-N); and 2) a nonhydrogen-bonded form. The latter one undergoes ESIPT to produce emission from two different molecular species, the normal form (N\*) and its tautomeric variant (T\*). The ratio of emission from the N\* and T\* species is sensitive to the dipole potential. Besides the dipole potential, other factors also affect the fluorescence of 3-hydroxyflavone dyes: 1) the relative contribution of the H-N species depends on the hydration of the membrane, that is, the ability of water to penetrate the membrane and form hydrogen bonds with the indicator; and 2) membrane hydration also leads to quenching of fluorescence from all three molecular species. In the case of F66, an increased dipole potential leads to a relative increase in the emission from species T\* at the expense of species N\*. However, these changes are confounded by less water quenching in lipid rafts, resulting in stronger fluorescence from raft-localized indicators from all three species. The dipole potential sensitive intensity ratio of 3-hydroxyflavone dyes is calculated as the emission in the low wavelength range divided by that in the high wavelength range. Both the N\* and H-N species contribute to emission in the low wavelength range. For F66, the emission ratio is expected to be negatively correlated with the magnitude of the dipole potential, that is, the emission in the low and high wavelength range should decrease and increase, respectively, in lipid rafts compared to the bulk phase. Because contributions of both the N\* and H-N species decrease in lipid rafts in the low-wavelength region, the emission ratio will change as expected. The behavior of PPZ8 differs in two respects: 1) it has hardly any emission in the low wavelength range, leading to potential problems related to background subtraction and consequent misestimation of the specific fluorescence intensity; and 2) the higher dipole potential and less hydration in lipid rafts are expected to change the relative contribution of the N\* and H-N bands in different directions. Because the emission from the dominating T\* band increases because of less water quenching in lipid rafts, the emission in the low wavelength range of PPZ8 should increase substantially, so that its emission ratio displays the expected positive correlation with the higher dipole potential in lipid rafts. However, the intensity in the low wavelength range of PPZ8 cannot increase strongly enough owing to points 1–2 above.

The magnitude of the dipole potential has usually been estimated to be in the range between 200 and 500 mV. Because the estimated difference between the dipole potential inside and outside lipid rafts is 50–100 mV, this effect is expected to have a significant impact on how membrane-related phenomena, for example, transmembrane transport, signaling, and membrane trafficking, involving membrane proteins occur inside and outside lipid raft microdomains.

Differences in the lipid composition and order of the membrane can influence the dipole potential and membrane-related phenomena not only under physiological conditions but also in diseases. The increase in the dipole potential associated with sphingolipid accumulation in an *in vitro* model of Gaucher's disease was found to be similar in magnitude to the difference in the dipole potential inside and outside rafts. Because alterations in the cholesterol and sphingolipid content of the membrane in a conditional glucocerebrosidase knock-out mouse model are comparable to those observed in the *in vitro* model we applied, the presented results imply that pathological changes in the lipid content of the cell membrane in human diseases in general, and in Gaucher disease in particular, can lead to significant changes in the dipole potential and can also substantially modify the functioning of membrane proteins, potentially contributing to the development of disease symptoms.

In conclusion, in the second phase of our experiments we have shown that the dipole potential is significantly larger in lipid raft microdomains than in the bulk membrane domain. The magnitude of this difference is large enough so that the conformation of the transmembrane domain of proteins is altered when they move into or out of lipid rafts. Consequently, some of the biological effects linked to lipid rafts can be attributed to the larger dipole potential present in this microdomain.

## 6. Summary

A largely unknown component of the membrane potential, the dipole potential, is generated by the ordered orientation of lipid carbonyl and membrane-attached water dipole moments. The dipole potential, generating an electric field much stronger than any other type of membrane potential, influences a wide array of phenomena including voltage-dependent conformational changes of the transmembrane domains (TMD) of proteins. Although activation of ErbB receptors (ErbB1-4) is driven by oligomerization mediated by intermolecular interactions, the TMD has been largely neglected in this regard. The dipole potential alters the conformation of transmembrane peptides, but its effect on ErbB proteins was unknown.

In our experiments, we showed using flow cytometric FRET and N&B analysis in confocal microscopy that the EGF-induced increase in the homoassociation of ErbB1 and ErbB2 and their heteroassociation are augmented by increasing the dipole potential. These effects were even more pronounced for ErbB2 harboring an activating Val → Glu mutation in the transmembrane domain (NeuT). The signaling capacity of ErbB1 and ErbB2 was also correlated with the dipole potential. Since the dipole potential decreased the affinity of EGF to ErbB1, the augmented growth factor-induced effects at an elevated dipole potential were actually induced at lower receptor occupancy. We concluded that dimerization induced by an elevated dipole potential will bring about TMD-driven receptor activation leading to increased signaling mediated by dimers stabilized by interactions among the extracellular domains and the TMDs. The dipole potential may play a permissive role in the clustering of ErbB receptors, and the effects of lipid rafts on receptor tyrosine kinases can be partially attributed to the dipole potential.

Theoretical considerations and indirect experimental evidence obtained in model membranes suggested that the dipole potential is larger in liquid-ordered domains believed to correspond to lipid rafts in cell membranes. Using three different dipole potential-sensitive fluorophores and four different labeling approaches of raft and non-raft domains we showed by quantitative confocal image analysis that the dipole potential is indeed stronger in lipid rafts than in the rest of the membrane. The magnitude of this difference seemed to be large enough so that the conformation of the TMD is altered when proteins move into or out of lipid rafts. The magnitude of this difference was similar to that observed between the dipole potential in control and sphingolipid-enriched cells characteristic of Gaucher's disease, pointing to a potential pathophysiological role for these findings. Our results established that the heterogeneity of the dipole potential in living cell membranes was correlated with lipid rafts and implied that alterations in the lipid composition of the cell membrane in human diseases can lead to substantial changes in the dipole potential.

## 7. Publikációk



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### List of publications related to the dissertation

1. **Kovács, T.**, Batta, G., Zákány, F., Szöllősi, J., Nagy, P.: The dipole potential correlates with lipid raft markers in the plasma membrane of living cells.  
J. Lipid Res. 58 (8), 1681-1691, 2017.  
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Total IF of journals (all publications): 28,122

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