Pores in lipid membranes and the effect of anaesthetics

Master's Thesis

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Abstract

Unmodified lipid bilayers exhibit a pronounced increase in transmembrane permeability during the lipid phase transition. This thesis addresses the issue of pore formation in protein -free lipid membranes in the phase transition regime. Differential Scanning Calorimetry is used to detect melting transitions in the bilayer membranes, whereas the Black Lipid Membrane technique, by applying transmembrane voltage, is employed to record transmembrane currents. Discrete current fluctuations, which are believed to be indicative for the existence membrane pores, are analysed using single ion-channel approach suited for processing protein-ion channel electrophysiological data. The work further investigates the dependency of the phenomenon on a number of thermodynamic variables, such as temperature, electrical potential and chemical potential. Special attention is given to the influence of anaesthetic-like compouds, such as octanol and etanol, on the membrane permeability. The basic concepts of thermodynamics are used to explain the fluctuating behaviour of the membranes and questions are raised regarding the interpretation of protein ion channel data in other studies.

"The most beautiful experience we have is the mysterious"

Albert Einstein

Motivation

It looks as if many of us have been using 'osmosis' as a convenient label without concerning ourselves with the mechanism. I too had assumed that in biological systems it was passive and related purely to size of 'holes' in the membrane versus size of molecules/ions.

On reflection I should have known better. During the Ph.D. school in Petroleum Engineering this summer I have learnt that clays in sedimentary sequences can act as semi-permeable membranes generating or retaining pressures greater than hydrostatic in the rocks beneath them. In this setting the clays do so by actively trapping ions in their inter-layer spaces. Hindsight says we shouldn't then be surprised that living systems achieve their results with such sophisitication and elegance.

Peter Agre and Roderick MacKinnon stand for decisive contributions to the biochemistry of cell membranes, but their discoveries also have an almost tangible aesthetic component. Their work has uncovered an amazing "economy of design" in the atomic structures of the water and ion channels that is breathtaking in its simplicity and perfection. Indeed, after seeing these molecular machines, you find yourself thinking, *"of course, this is how it must be, this is how it must work! What more could we ask of science?".*The biochemical basis for the transport of water – the most abundant and primordial substance of life – and ions – these tiny, mundane and yet absolutely essential constituents of the living world – can now be understood in unparalleled detail. The molecular control of membrane permeability by membrane proteins, among many other functions, have been intensively investigated over the past years. However, the physical fundamental understanding for many membrane-related mechanisms, including membrane mediated transport, is not yet fully understood. This becomes even more inexplicit in the light of reports from the 1980's, which pointed out to the fact that protein-free lipid membranes can become permeable under certain conditions of temperature and voltage.

With the field rich in Nobel Prize winners (Erwin Neher and Bert Sakmann in 1999, Peter Agre and Roderick MacKinnon in 2003) and a sense of ambiguity in sole lipid membranes permeation phenomenon, which exert interestingly similar features to the membrane protein-driven permeation, electrophysiologically-wise, the motivation to explore the concept in more detail does not seem to require more justification.

Anaesthesia, o the other hand, even though as a medical procedure it has been used as an indespansable part of clinical practice for centuries, is a phenomenon, which many scientists and physicians have become puzzled over for equally long time. The process, which every year causes millions of patients to

reversibly lose the most precious human attribute - consciousness - and become insensitive to pain, has not been yet resolved on a molecular level ever since an anaesthetic action of simple molecules such as ether or chloroform was recognised in the half of the ninetieth century. The Meyer-Overton theory, which came few decades later and correlated the potency of a drug with its solubility in oil posed a long list of questions among molecular biologists: How could such a structurally diverse group of drugs, from simple inert gases, as xenon, to complex steroid compounds produce a common end point? Are there then common mechanisms underlying the action of anaesthesia? Do anaesthetics act specifically on ion protein channels or interact unspecifically with the lipid membrane, as Meyer and Overton predicted? With a great expansion of ion channel field contemporary to the upstanding mystery of anaesthesia, the protein-targetted anaesthesia has been taken as a more plausible approach, with no convincing explanation though. With a great number of protein channel candidates, consistent picture of how anaesthesia might work was only found for a handful of them. This is usually judged from a change in the electrophysiological recordings for membrane patches with an anaesthetic component. If, however, pure lipid membranes can resemble responses of the protein channels activity electrically, could the anaesthesia effect also be similar in this case? The curiosity is obvious.

The simplicity of the idea and the lack of trust in the importance of the phenomenon from biologists are additional driving force to investigate the problem more closely. Solid thermodynamical description of the process speaks additionally for the logics behind it. The fact that the membrane transport can be considered one of the defining principles of life, the basis of cellular and organismal physiology, make it challenging and worth exploring even if it was to take late hours experimenting and massive amounts of coffee consumed.

Objectives

The primary objectives of the study were to:

1. Rediscover the findings from the 1970s' indicating that the protein-free membranes show channel-like events in their melting transitions.

2. Explore the permeability of the lipid membrane in a thermodynamic context.

3. Try to see how such hypothetical lipid channels would respond to a presence of simple anaesthetic compounds, such as octanol and ethanol, in lipid membranes.

The first chapter gives a brief introduction to the concept of biological membranes and the interesting phenomenon of lipid melting. The second chapter sets the scene for theories of pores in membranes, including pore forming proteins, and a historical background for lipid ion channel discoveries. A thermodynamic description of fluctuations, which give rise to the observed processes is also given together with the anaesthetic-induced depression of the temperature of the melting transition, which allows for describing the phenomenon of anaesthesia on lipids in a quantitative manner. Finally, after the principal materials and methods employed in the work are introduced in chapter three, the main outcomes of the research are presented in the Results section. The last chapter summarises what it has been observed and how it might contribute to the overall understanding of the problem.

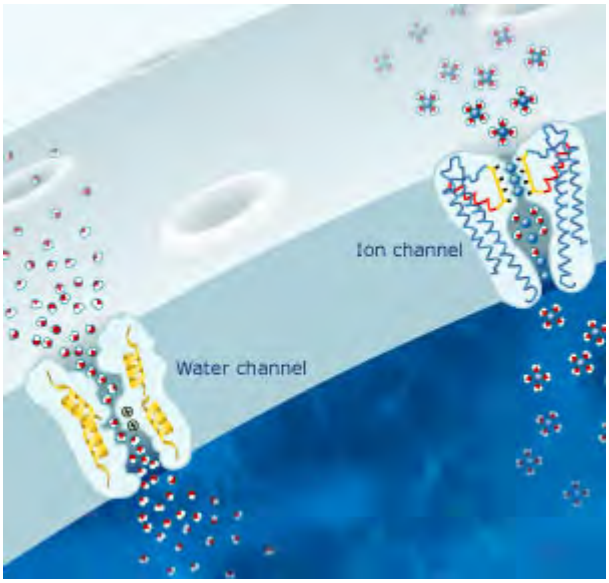


Figure 1: Water channels and ion channels have been the two key players in the ion-channel field since 2003, when Peter Agre and Roderick MacKinnon received a Nobel Prize in Chemistry *'for discoveries concerning channels in cell membranes'*. Picture taken from *www.nobelprize.org.*

Contents

1 Introduction

Excitation and electrical signalling in the nervous system involve the movement of ions through ion channels - this line appears in every textbook of molecular biology nowadays. The Na^+, K^+, Ca^{+2}, and Cl^- ions are believed to be responsible for almost all the action. Each channel is widely regarded as an excitable molecule, as it is specifically responsive to some stimulus: a membrane potential change, a neurotransmitter or other chemical stimulus, a mechanical deformation etc.

Frederick Engels wrote in 1883 that "*life is the mode of existence of protein bodies*", which seems to have become an idea adopted by many physiologists nowadays, in attempting to resolve the structures of common ion channel proteins and thereby explain their prominent function in the permeability of a lipid membrane.

Starting from the 1970's however, physicists turned their attention to the lipid matrix itself and the evidence of its permeation without the presence of proteins has remained a mistery ever since. The exact mechanism of lipid pore formation and its biological relevance next to protein ion channel activity has been still longing for an answer, although the uniform laws of physics suggest inevitable truth.

For a new entrant to the world of biophysics the problems is extremely interesting. All cells, whether bacterial, plant, or animal, are enclosed by membranes, the basic components of which are lipid bilayers. The cell membrane ultimately acts as the defining principle of what constitutes a cell and what constitutes the rest of the world. Lipid bilayers are semipermeable: small uncharged molecules can pass more or less freely from one side of the membrane to the other, but for charged species or macromolecules, such as proteins and DNA, the lipid bilayer is a major obstacle to diffusion. The transmembrane transport has been assigned to a part of the family of membrane proteins referred to ion channels, which are specialized in mediating the transport of charged species and macromolecules across the bilayer.

Lipid pore formation, which has proved to be an observable fact for almost three decades now, is undoubtly one of the mechanisms that can contribute to the overall efectiveness of transport mechanisms across the membrane. On close analysis, it shares the principal characteristics of protein ion channel electrical activity, measured with patch-clamp techniques by thousands of biologists every day. The generation and maintenance of the internal structures and integrity of cells depends largely on the effectiveness of the membrane in keeping the inside in, and the outside out. Therefore, it is necessary to examine the problem of pure lipid membrane perameability more closely in order to gain a full picture

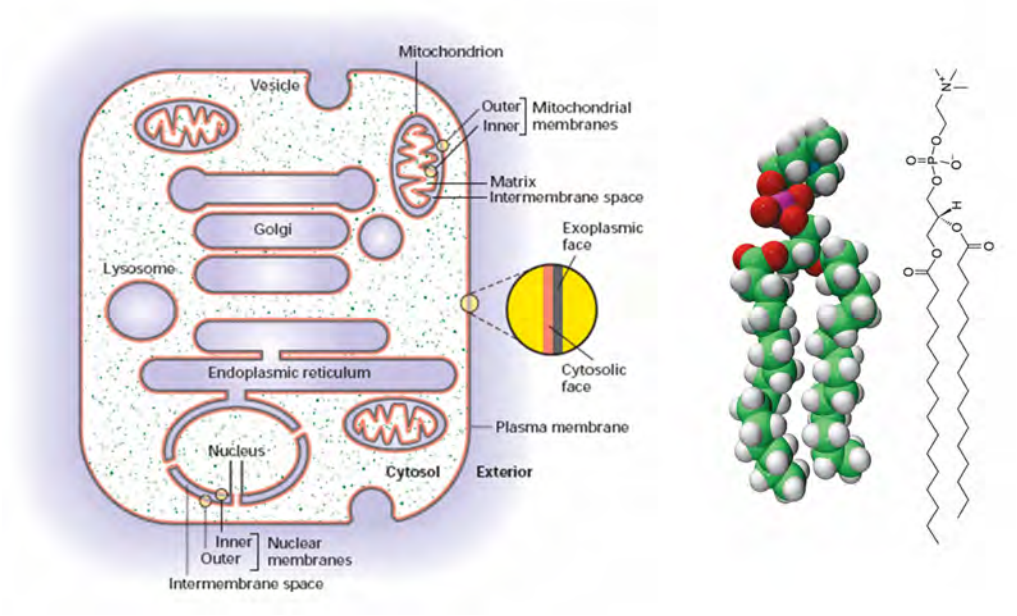


Figure 2: A biological membrane is a common feature each eucaryotic cell (*left*). Different types of organelles and smaller vesicles are enclosed within their own distinctive membranes, which carry out specialized functions and take part in gene expression, membrane synthesis or intracellular transport (*Lodish et al.*, 2003). Single phospholipid (*right*) is an amphiphilic molecule - with hydrophilic head group and two apolar hydrocarbon chains (here: picture of a DMPC, *www.avantilipids.com*). Despite being only 5nm thick, biological membranes are considered as perfect isolators.

of the mechanisms used in the transfer of ions and macromolecules across the cell membrane.

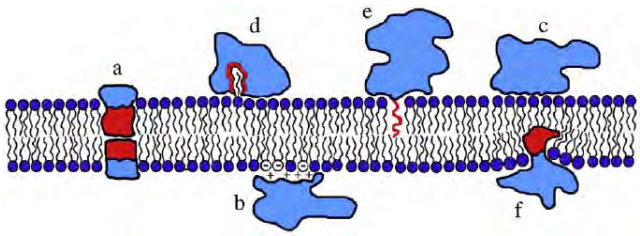


Figure 3: Membrane proteins can bind to, anchor to, imbed in, and penetrate lipid membranes. (a) trans-membrane-spanning amphiphilic peptide dimer; (b) electrostatic binding; (c) non-specific binding by weak physical forces; (d) anchoring via a lipid extended conformation; (e) anchoring by an acyl-chain anchor attached to the protein; (f) amphiphilic peptide partially penetrating the bilayer. Transmembrane proteins may belong to huge protein ion channel families (*Khandelia et al.*, 2008).

1.1 Biological membrane

A biological membrane encloses every cell, defining the cell's boundary and providing a barrier between its contents and the environment. It is considered as a highly selective filter and a device for active transport. It controls the entry of nutrients and the exit of waste products, and it generates the differences in ion concentration between the interior and exterior of the cell. The plasma membrane also acts as a sensor of external signals, allowing the cell to change in response to environmental cues.

All biological membranes, plasma membranes and membranes of integral organelles, share a common general structure - they are assemblies of lipid and protein molecules held together by mainly non-covalent interactions. From the experiment by *Gorter and Grendel* (1925) on membranes from red blood cells and follow-up electron microscopy and X-ray diffraction studies conducted in the 1930's a double-layer basis for the cell membrane structure was established. Proteins on the other hand are nowadays believed to be arranged in varous ways in this lipid matrix of just 5nm thickness (fig.3). Despite the fact that the lipid bilayer is very thin, it is regarded as impermeable barrier to the passage of most water-soluble molecules and ions (fig.4). The permeation of solutes across the membrane is mainly due to single protein molecules spanning the membrane.

1.2 From Singer-Nicolson to lipid rafts

In the first model of membrane structure, proposed by *Singer and Nicolson* (1972) and termed the '*fluid mosaic model*' of the membrane, membrane proteins were seen as a heterogeneous set of globular molecules partially embedded in a phospholipid matrix, which could be loosely compared to randomly dis-

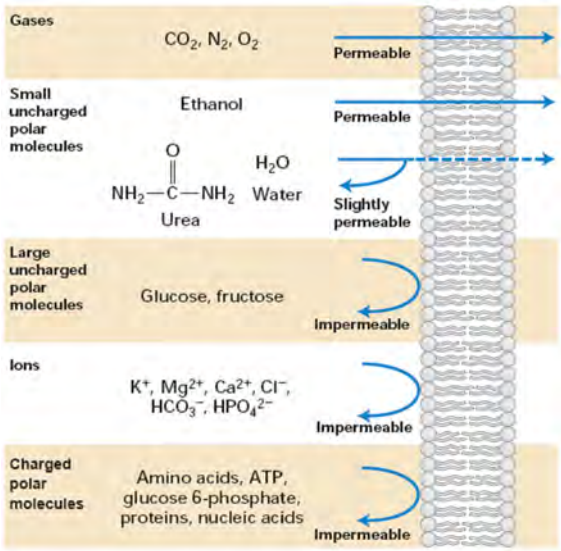


Figure 4: A lipid bilayer is permeable to small hydrophobic molecules and small uncharged polar molecules, slightly permeable to water and urea, and essentially impermeable to ions and to large polar molecules at appreciable rates by passive diffusion. Classical picture from biological textbook (*Lodish et al.*, 2003)

tributed islands floating in the lipid bilayer sea. The bulk of the phospholipid was believed to be organized as a discontinuous, fluid bilayer, although a small fraction of the lipid might have interacted specifically with the membrane proteins, and, in addition, the proteins could have formed protein clusters. The Singer-Nicolson model had served as fundamental model of membrane structure for over three decades until Gunther Blobel's pioneering work on the mechanisms proteins integrate with the membrane (which he was awarded a Nobel Prize in Medicine in 1999). His observations suggested that protein structures are able to move about in the plane of the membrane, which, at the same time required that the fluid mosaic model had to be refined.

Today, membranes are believed to be more mosaic than fluid (*Engelman*, 2005), i.e. they are visualized as heterogenous, dynamic lipid-protein structures, where membrane lipids can exist in different states and form so-called domains, giving the membrane a compartmentalised character. Lipids themselves now appear to play a more active role in the membrane than previously believed, in addition to providing a solid support for membrane-embedded proteins. In many cases they are reported as crucial elements in triggering biochemical reactions in the cell, which is often referred to as lipid signalling (*Yakimova et al.*, 2006; *Eyster*, 2007) and they have been shown to have a considerable effect on protein ion channel activity (see part in *section 2.3*). Furthermore, lipid clustering in domains and the increased fluctuations at domain interfaces during the phase

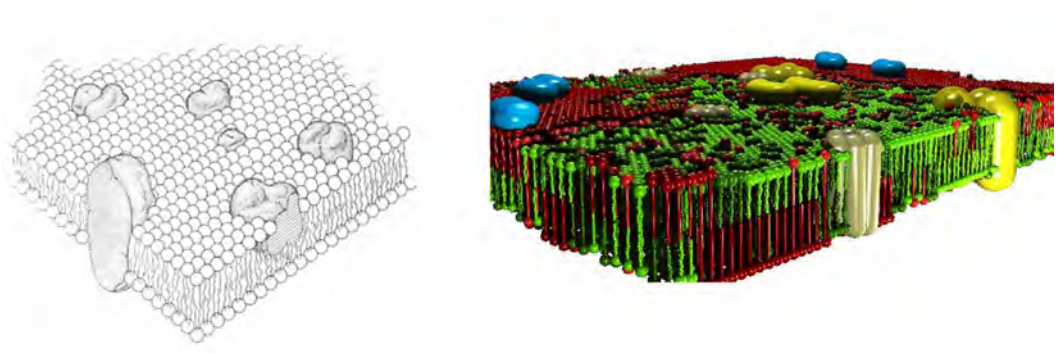


Figure 5: Fluid mosaic model of membrane stucture (*left*, (*Singer and Nicolson*, 1972)) and a modern picture of biological membrane (*right*, (*Seeger*, 2006)) with lipid domains in solid-ordered (*red*) and liquid disordered (*green*) state. The lipid composition of the two monolayers might vary (*Devaux*, 1991). Membrane proteins can aggregate and interact with the lipid components in various ways, altering physical state of the membrane.

transition have turned out to be of great importance in determining permeability of protein-free lipid membranes.

1.3 Lipid melting

One of the most prominent characteristics of lipids, and thus protein-free bilayer membranes, is their ability to undergo reversible phase transition from a crystalline-ordered state at lower temperatures to a liquid-disordered state at higher temperatures (fig.6). Such transitions were first characterised in phospholipids (*Steim et al.*, 1969; *Mabrey and Sturtevant*, 1976) before they were found in biomembranes (*Reinert and Steim*, 1970; *Melchior and Steim*, 1976) and they were mainly detected by the upstanding DSC technique (see section *3.2.1*). The thermodynamic description of the phenomenon also dates back to the early 1970's when *Hinz and Sturtevant* (1972) and *Steim et al.* (1969) first observed that melting transition has cooperative character and furthermore, that increased unsaturation in fatty acid residues might result in lowered lipid transition temperatures.

Today, we know that transitions of biological membranes usually happen around $15K$ below body temperature (*Heimburg and Jackson*, 2005), and lipids extracted from biomembranes can undergo melting transitions over a very wide ranges of temperatures, from $-20°C$ to even $60°C$. Furthemore, from calorimetric measurements it has been shown that proteins can significantly affect melting transitions of lipids (*Ivanova et al.*, 2003; *Oliynyk et al.*, 2007) and the melting transition of lipids alone depends on intristic properties of lipids such as lipid chain length or interactions of lipid head groups with an aqueous medium.

By assuming that lipids can only be found in two states, a fluid disordered state and a solid-ordered one, their melting point T_m can be defined as the temperature at which the occurrence of the two phases is equally probable. Thus, at the melting transition the free energy difference at equilibrium of the fluid-state lipid and gel-state lipid is zero:

$$\triangle G = G_o - G_s = 0$$

and knowing that

$$\triangle G = \triangle H - T_m \Delta S$$

where $\triangle H$ and $\triangle S$ denote melting enthalpy and entropy, we arrive at

$$T_m = \frac{\triangle H}{\Delta S}$$

The changes in physical properties of lipids at the phase transition temperatures may include a change in membrane thickness (*Pagano et al.*, 1973; *Antonov*

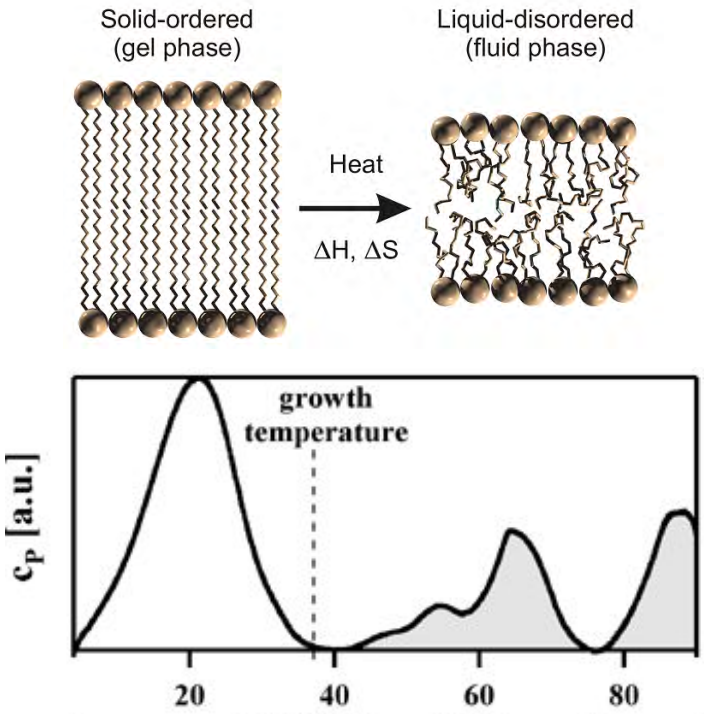


Figure 6: *Top*: Lipid phase transition stands for lipids undergoing from solid-ordered to liquid-disordered state at increasing temperatures. This causes an increase in both the enthaply $\triangle H$ and the entropy $\triangle S$ of the system, accompanied by an increase in area and reduction of lipid bilayer thickness by $\sim 20\%$. The overall membrane volume changes only by $\sim 4\%$ during the transition (*Heimburg and Jackson*, 2005). Picture courtesy of (*Blicher*, 2007). *Bottom:* Change in heat capacity of membranes from *E.coli* during the melting process (*Heimburg and Jackson*, 2005). At the melting temperature, the heat capacity reaches a maximum. The lipid melting takes place slightly below the growth temperature of $37°C$, the higher temperature peaks represent the unfolding of membrane proteins.

et al., 2003; *White*, 1975) or increased membrane permeability and thus, ionic conductance, as first reported by (*Antonov et al.*, 1980, 2005), and later also confirmed by others (*Bogatyreva et al.*, 1998). *Gudmand* (2008) showed that an enzyme phospholipase A_2 exhibits enhanced activity on lipid domain membranes when they are in the melting transition temperature regime. *Pan et al.* (2008); *Chu et al.* (2005) observed decreased membrane bending rigidity and increased membrane compressibility, studies of *Bogatyreva et al.*, (1998) confirmed lowered membrane stability, which would favour pore formation in the membrane in the melting transition. These and many other response functions (isothermal area and volume compressibilities, bending elasticity or relaxation times) are closely related to the heat capacity changes, with their maximum at the melting point temperature. This can be rationalized thermodynamically with the proportional relation between enthalpy and volume/area changes close to the melting transition (*Heimburg*, 1998) - see also *section 2.4*.
On the other hand, many intensive thermodynamical variables, such as changes of pressure, chemical potential or electrical potential, which determine the overall entropy of the membrane, can induce a change in lipids phase transition and, in turn, alter macroscopic properties of the membrane (see *section 2.4*).

2 Membrane permeability

2.1 Pore theories

"[...]different membranes [may] have different sizes of holes. Or a membrane may allow certain substances to pass through it because of their solubility in the substance of which the membrane is composed. Or, thirdly, they may possibly form reversible chemical compounds with the substances to which they are permeable [...]"

William Bayliss 'Principles of General Physiology', 1918

It was not until 1950's that biophysicists universally accepted that ion channels are pores spanning the membrane lipid bilayer.

Nevertheless, the pore hypothesis for biological membranes has been discussed since 1843, when Ernst Brücke (*Brucke*, 1843) first described the phenomena of osmosis through the investigation of diffusion across animal membranes. He suggested that microscopic, fluid-filled spaces in the membrane can be considered a "system of capillary tubes" (*Kanäle*) across the membrane which would allow a stream of water flow down its concentration gradient (osmosis) and a flow of only a small stream of solute in the opposite direction. More precisely, water in Brücke's theory would form a mobile boundary layer lining the walls and if the pore is narrow enough, meaning that there is only a room for the boundary water layer lining the walls, then the water stream could flow through the pore. The wider pores (more than three water molecules in diameter), would also allow the flow of solute through the membrane in the opposite direction to water molecules, i.e. the membrane will additionally show some permeability to solutes. Brücke was the first to propose the idea of aqueous pores and to indicate that their selectivity could depend on the pore molecular dimensions. His "pore theory" also quickly became a standard basis for further discussions of osmosis and secretion.

The importance of the Brücke's pore theory started to be noticed by many other famous physiologists: Carl Ludwig (*Ludwig*, 1852) a few years later suggested that the pores are essential in the secretion process of urine, where according to his theory urine was an ultrafiltrate of blood serum, forced by the blood pressure through capillary walls of the glomerulus. Adolf Fick, based on the Brücke's pore theory proposed the diffusion equation to describe a flux for pores with a mobile boundary layer of water (*Fick*, 1855). E.W. Reid, almost half a century later, having summarized the Brücke-Ludwig-Fick contribution to 'pore diffusion theory', proposed a molecular pore model supporting the membrane semipermeability and osmosis (*Reid*, 1898) and William Bayliss in the

1960's (*Bayliss*, 1959) discusses the mobility of ions in aqueous solution and points out the inverse relation between the atomic number and the friction of the ion, the latter defined as the degree to which the ion is hydrated. The concept that the least hydrated ion is the most mobile was later supported by Leonore Michaelis (*Michaelis*, 1926), who apart from having explained the enzyme kinetics together with Menten, also suggested that an electric charge on the walls of the channel might be a factor governing the pore's selectivity to ions. The work of Michaelis was further often regarded as a theoretical basis for biological pore theories and referred to as 'the pore theory of Michaelis' in literature.

It was not until almost three decades later when, in addition to mechanical size of the ion, the interaction energies in determining the membrane ion permeability were also concerned. Mullins et al. (*Mullins*, 1961) proposed that the barrier to move heavily hydrated ions into a narrow pore is the energy required to dehydrate the ion. This energy barrier can be lowered if the water molecules are replaced by 'solvation of similar magnitude obtained from the pore wall'. In this respect, the previous theories of membrane pores, where permeability would depend on the size of the rigid-like ion-water complex, were modified. The pore size was suggested to be equal to the size of the crystal diameter of a specific ion plus the diameter of one water molecule $2.72\mathring{A}$, so that the pore walls fit closely to the ion-water molecule being transported and thus provides its salvation. Ions not fitting closely are not sufficiently solvated and therefore cannot enter the pore.

This concept of membrane permeability with solvated ions passing through membrane pores prevails also today among membrane biologists. The introduction of radioactive traces and the voltage clamp further allowed the questions about the mechanism of the membrane transports to be addressed. All the possibilities cited by Bayliss in the front page of this thesis turned out to take place and different 'pore' and 'carrier' transport proteins inserted into the lipid matrix are now regarded as indispensable factors affecting membrane permeability.

Let's have a closer look on what it is known about ion channels today and how the concept of non-protein ion channels emerged, in pure lipid membranes.

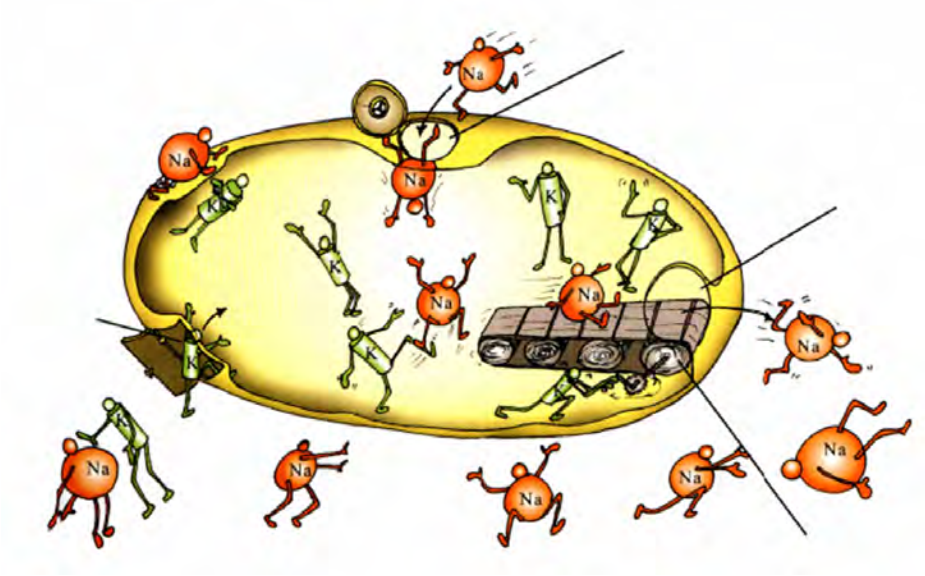


Figure 7: Hodgkin and Huxley in 1952 (*HODGKIN and HUXLEY*, 1952) used the concept of voltage-operated ion channels to provide the basis for an understanding of the nerve action potential. By 1970 terms Na^+- and K^+-channel were in frequent use, even though no direct evidence for the existence of channels was available from electrophysiological preparations. The picture represents a Na^+/K^+ pump, which unlike the sodium and potassium ions individually, is an example of active transport of molecules across a membrane. In the Na^+/K^+ pump, the active transport is coupled to ATP hydrolysis to obtain enough free energy to transport the ions against their concentration gradient: for every ATP molecule used, three Na^+ ions are pumped out of the cell and 2 K^+ ions are pumped into the cell (adapted from (*Pollack*, 2001)).

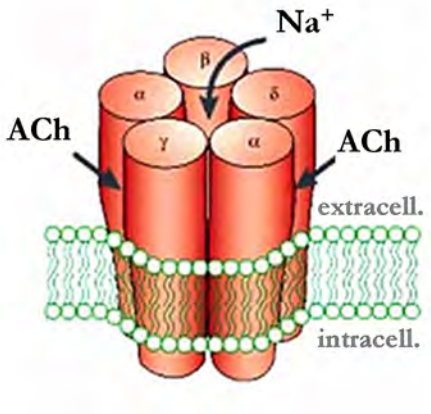


Figure 8: Channels associated with neuronal nicotinic acetylcholine receptor (AChR) and have been one of the most extensively studied ion channel proteins already since the 1970's, when Neher and Sakmann first performed single ion channel analysis on ACh-induced currents from frog muscle fibres (*Neher and Sakmann*, 1976). Later on the stucture-function relationship of the protein was established *A et al.*; *Unwin*, 1999, which has served as a model of action for other ligand gated ion channel proteins. Figure shows subunit composition of one of the neuromuscular AChR subtype. (*http://www.nuk.usz.ch*).

2.2 Ion channels

- barrels spanning the membrane

"Ion channels are defined as pores in the membrane that regulate the flow of ions across the normally insurmontable barrier of the lipid bilayer" - it can be read from the Encyclopedia of the Neurological Sciences (*Aminoff and Daroff*, 2003). This function is by default assigned to membrane-spanning proteins, which by converting from shut (nonconducting) to open (conducting) conformations let the ions through the membrane. It is regarded a fundamental truth especially in nerve conduction theories. The flow of ions is the source of the electrical current that changes the potential difference across the membrane, which in turn is very convenient to be measured by various electrophysiological means.

Protein ion channels are structures with a hole down their middle that allow the movement of ions across the supposedly impermeable cell membrane and thereby control an enormous range of biological functions, from signalling in the nervous system, through coordination of contraction, particularly in the heart (sodium, calcium and potassium channels (*Terrenoire et al.*, 2007)), to transport in most cells and organelles. Information that is transmitted across cell membranes might moreover contribute to cell-to-cell signaling processes as ions and proteins crossing cell membranes, may trigger various intracellular

signaling cascades (*Keurs and Boyden*, 2007). Lastly, ion channels are also considered major targets for therapeutic intervention in the treatment of many disorders, with binding sites for antiepileptic agents, antipsychotics, anxiolytics, local anaesthetics, antihypertensives or antiarrhythmics, to name a few.
All in all, protein ion channels are of central importance to both electrophysiologists and clinical scientists nowadays and they have become a reference point in understanding the cell physiology and improved treatment of many channel-associated disorders (termed channelopathies *Brugada et al.* (2007)). Recent reports on the suggestions for the existence of ion channels in plants controlling gas exchange with the atmosphere (*Serna*, 2008) or the indication for the odorant-gated channels in insects (*Sato et al.*, 2008; *Wicher et al.*, 2008) could further serve as the proof for the vastly developed ion-channel oriented thinking in medicine and biology.
Protein ion channels are usually pictured as barrels or corks embedded in the membrane matrix with lots of architectural motifs such as hinges, swivels and switches sticking out which are referred to selectivity filters or protein residues that constitute gating and thus modulation of the binding activation sites.

Ion channels exhibit two basic properties: permeation and gating.

- **Permeation** refers to the ability of an ion to pass through an open pore from one side of the membrane to the other. It can be either selective or nonselective, i.e. permeation properties of a channel specify how well the various types of ions are conducted through the pore and thus determine whether a channel is excitatoty or inhibitory. Protein ion channels conduct specific ions at very high rates, for instance $10^7 - 10^8$ potassium ions per second flow through a K^+ channel, which essentially excludes Na^+ ions, meaning that the channel is selective for potassium; Nicotinic acetylcholine receptor can serve as an example of ligand-gated and nonselective ion channel. *Fig.*10 explains how the relative size of K^+ and Na^+ ions vs. size of the gate determines the potassium channel's selectivity to these ions (Roderick MacKinnon, Nobel Prize, 2003).

- **Gating** refers to the process of switching between between open and shut states of the channel. By opening and closing a gate, similarly to a door, conduction of a channel is turned on or off. The process can be regulated by an external stimulus such as ligand binding, mechanical stress, intracellular second messengers (such as Ca^{+2} or cyclic nucleotides like cAMP and cGMP) or membrane voltage. The channels are called ligand-binging-, mechanosensitive, second-messenger-gated or voltage-gated ion

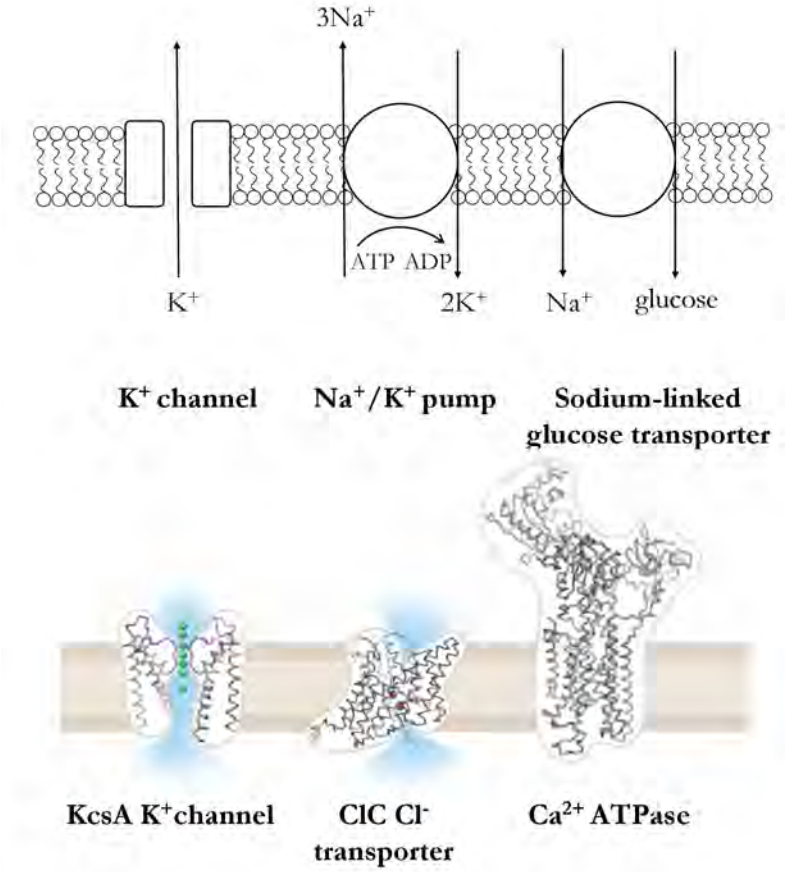


Figure 9: The major classes of membrane ion channel proteins (*top*) and the corresponding examples with crystallographically defined architectures (*bottom*, (*Gouaux and MacKinnon*, 2005)). Single ion channels, such as K^+-channel, usually transport channels passively across the membrane, along their concentration gradients. Bigger protein complexes pump molecules or ions through a membrane against their concentration gradient, requiring an external source of energy. Arrangement of ion-binding sites relative to the position in the membrane is believed to determine the ion conduction rate.

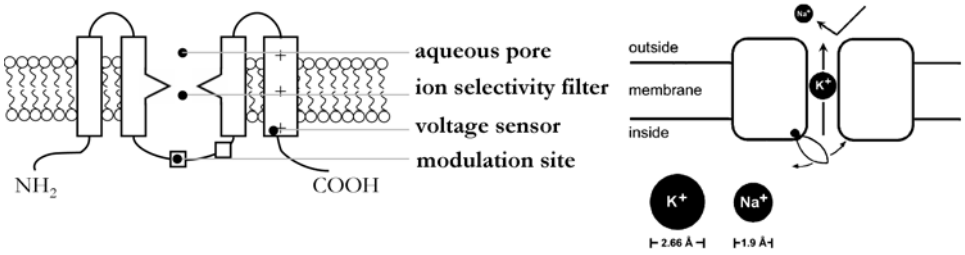


Figure 10: Conductivity, selectivity and gating as the main functional characteristics of protein ion channels. The experimental observations are found to go along with the structural details of the channel (*left*). For instance, despite the smaller atomic radius of Na^+, only K^+ ions can get through the pore of the potassium ion channel (*right*). More energetically favourable interactions of potassium ions to carbonyl oxygens facing the pore than sodium, would serve as an explaination (*Gouaux and MacKinnon*, 2005).

channels, respectively. Apart from many stimulating factors, there exists also many which were proved to inhibit the channel activity (i.e. anaesthetic compounds).

The net flow of ions through the pore is determined by a balance of diffusion from high to low concentration together with the drift of ions in the elelctric field across the membrane. At equilibrium, the two forces balance out, which results in no net current flow through the membrane. A membrane voltage at which there is no net flow of ions from one side of the membrane to the other is called the reversal potential (or Nernst potential) of a particular ion and it is described by Nernst equation:

$$E_0 = \frac{RT}{zF} \cdot ln \frac{[A]_o}{[B]_i}$$

where z- charge of an ion, F- Faraday constant, T-temperature, R-gas constant and $[A]_0$, $[B]_i$ are the extracellular and intracellular concentrations of ions of interest with permeability P_i.
When a channel type that is selective to one ion species dominates within the membrane, the voltage inside the cell will equilibrate to the reversal potential for that ion. However, the identity between the terms reversal potential and equilibrium potential is only true for single-ion systems. In multi-ion systems, where the cell has significant permeabilities to more than one ion, the cell's

resting potential can be calculated from the Goldman-Hodgkin-Katz equation rather than the Nernst equation:

$$E_R = \frac{RT}{zF} \cdot ln \frac{P_K \cdot [K^+]_o + P_{Na} \cdot [Na^+]_o + P_{Cl} \cdot [Cl^-]_i}{P_K \cdot [K^+]_i + P_{Na} \cdot [Na^+]_i + P_{Cl} \cdot [Cl^-]_o}$$

assuming that the intra- and extracellular concentration of ions (Na^+, K^+, Cl^-etc.) and their permeabilities are known. The reversal potential is usually provided by current-voltage relationship for an ion channels under consideration (see *fig.34* and *section Appendix*).
All in all, the ionic current I_m across a membrane can can be calculated from that ion's conductance g_I and the driving force, which is represented by the difference between the membrane resting potential E_R and the ion's equilibrium potential E_0:

$$I_m = g_I \cdot (E_0 - E_R)$$

From this it follows that the ionic current will be zero if the membrane is impermeable to the ion in question, regardless of the size of the driving force.

One can find extensive experimental literature reporting the current flowing through a single channel molecule, over a range of voltages and concentrations of different types of ions. The current through an (already) open channel determines important biological properties of channels and thus cells, and tissues. The amount and type of ionic flux (e.g., carried by Na^+, K^+, $Ca^{2+}$and/or Cl^- ions) seems to be well understood in terms of characteristics of a channel type, with however little concession to physical phenomenon underlying the manner in which opening and closing of channels works. Why do the current recordings of the active channels show many subconductance states that are almost ideally rectangular in shape? It could mean that the ion channel protein changes its conformations in a non all-or-nothing fashion, which is a rather unlikely scenario. Are the electrostatic interactions of ions with the protein residues lining the pore wall sufficient in explaining ion channel selectivity and gating? *Berneche and Roux* (2001) from their molecular dynamic energy simulations show that the magnitude of the fluctuations of the carbonyl oxygen atoms, that form the selectivity filter is much greater than the difference in the radius of Na^+ and K^+ ions, which indicates that selectivity cannot be judged only based on a simple geometry and electrostatic interactions. *Sachs and Qin* (1993), on the other hand, have recorded current traces from gigaohm seals between patch pipettes and silicon rubber surfaces, which occured as quantised, channel-like steps *"indistinguishable from the gating of biological ion channels"*.

In the view of these findings, it seems reasonable to suggest that the other fundamental component of the membrane - lipid matrix - also needs to be taken into account when considering ion channel activation. Physics, to be more precise the second law of thermodynamics, tells us that there is always a certain probability for a process to occur, be it for instance flow of solutes across the protein-free lipid matrix. The entropy of the system, which governs the likelihood of a process occuring, depends on many physical variables like temperature, pressure or chemical potential of the compounds of the system. In this view, understanding and accepting general mechanism of protein ion channels action gets even more complex and drawing conclusions is no longer feasible.

Investigation of ion transport/membrane permeabiliy seems to be much simplified when analysing protein-free lipid membranes. Influence of only a few, purely physical parameters can be tested, all of which are related by a uniform second law of thermodynamics.

Figure 11: Single-ion channel current recordings of acetylcholine receptor measured by Neher and Sakmann in 1976 initiated a large expansion of ion channel field research among biologists (*Neher and Sakmann*, 1976).

2.3 Lipid ion channels

- History of investigation

Before moving on to thermodynamic description, let us summarize briefly how the studies on permeability of pure lipid membranes proceeded, parallel to the expansion in protein-ion channel field.

Yafuso et al. (1974) as one of the first in the field studied protein-free lipid membranes in voltage clamp conditions and reported spontaneous and multilevel conductance changes, which they attributed to channels that formed in the film. Only two years later, Neher and Sakmann first performed the patch clamp single-channel analysis on protein ion channels from frog muscle fibre membranes (*Neher and Sakmann*, 1976), for which they both were awarded a Nobel Prize in Physiology and Medicine in 1991. That was considered a breakthrough in cell's physiology, which had put the lipid ion channel story in the shade.

However, the concept of pores in pure lipid membranes continued to develop amongst physicists at its own pace. *Antonov et al.* (1980) reported the appearance of single ion-channel-like events in pure lipid membranes of DSPS at the melting temperature of the lipid (*fig.12*). He further named the process 'soft perforation' (*Antonov et al.*, 2005) of lipid membranes, in contrast to electroporation of membranes, i.e. poration induced by high electric fields. At the same time he excluded the possibility that the phenomenon was caused by impurities in the membrane (as many had remained sceptical about (*Goegelein and Koepsell*, 1984)), as the fluctuations appeared with reversible membrane temperature change across its melting transition, which would wash any impurities away.

Experiments by (*Kaufmann and Silman*, 1980; *Boheim et al.*, 1980; *Yoshikawa et al.*, 1988) also supported the idea that transmembrane ionic currents observed close to lipid melting transitions could account for pores in lipid membranes without the involvement of proteins. They showed that lipid melting transi-

tions and resultant discrete current fluctuations can be induced by applying suction or a transmembrane voltage to membranes under voltage clamp condictions. The suggestion that a transmembrane voltage could move the lipid phase transition was also made by *Antonov et al.* (1990) and *Bhaumik et al.* (1983) few years later (see also *section Appendix*).

Kaufmann and Silman (1980) turned their attention to the influence of pH, yet another thermodynamic variable, on the melting bahaviour of lipids with respect to ion channel fluctuations. In 1989 *Kaufmann et al.* (1989) gave a thermodynamic picture on how various intensive thermodynamic variables, such as temperature, voltage, lateral pressure of pH change are coupled to influence the channel fluctuation phenomenon in pure lipid membranes.

Extensive literature on permeability studies of pure lipid membranes exists, but appears to have been largely underestimated by the scientific protein-channel-oriented community.

Protein-lipid interactions

There are already many studies published which cite the importance of lipids for the proper function of protein ion channels. *Cannon et al.* (2003) report that they are required for the refolding of the bacterial KcsA potassium channel to a functional tetramer form. Since the protein is supposed to undergo a conformational change when the channel opens and closes, the suggestion has been made that lipids might also influence channel gating mechanism function (*Valiyaveetil et al.*, 2002).

The phospholipid surface charge was found to influence ion conduction in the K^+ channel over two decades ago (*Bell and Miller*, 1984) and the peptide influence on lipid membrane local properties has also been studied in our lab. It has been shown that the fluctuations of lipid membranes in the gel state at a fixed temperature are increased in the presence of protein in the membrane, which was interpreted as if a certain fraction of lipids were in the liquid state. This was further supported by the calorimetric data, which showed a shift of melting profiles of lipid-protein complexes to lower temperatures compared to protein-free membranes (*Ivanova et al.*, 2003). Melting of some fraction of lipids at lower teperatures was found to be the reason for fluid nanodomains present close to peptide-induced pores detected by AFM (*Oliynyk et al.*, 2007). Similar conclusions were also drawn from Monte-Carlo simulations (*Heimburg and Biltonen*, 1996).

All in all, lipid-protein interactions do play a role in the behaviour of both parties - peptides affect the local thermodynamic state of the system, but also the state

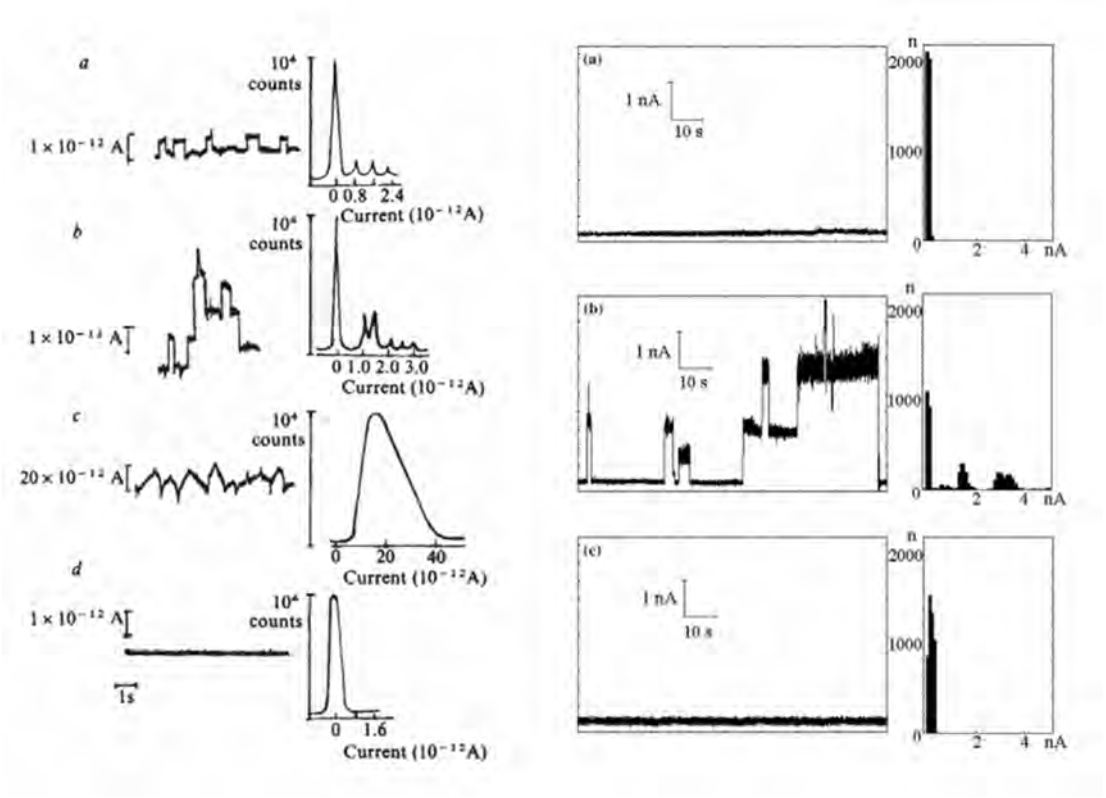


Figure 12: First observations of ion channel like fluctuations in pure lipid membranes. *Left*: current fluctuations from DSPC membranes recorded at the melting temperature $59°C$ (*a*), below the melting temperature (*c*) and above the melting temperature (*d*) and the step-wise current changes for membranes from egg-lethicin (*b*) (*Antonov et al.*, 1980). *Right*: Similar findings for DPPC membranes (*Antonov et al.*, 2005): *a*-above melting temperature of $41°C$, *b*-at melting temperature, *c*-below melting temperature. Amplitude histograms next to current traces show the current amplitude distribution and indicate the discrete character of current changes.

of the membrane can be a regulating factor for the action of protein molecules, by influencing their capability to form transmembrane pores (*Khandelia et al.*, 2008).

Abundant evidence also exists that the lipid bilayer composition might affect the function of some integral proteins, such as pumps (*Lee*, 1998) and membrane thickness has a direct effect on channel conductance probably due to the changes in hydrophobic mismatch, as reported for gramicidin A channels (*Martinac and Hamill*, 2002) or potassium ion channel (*Yuan et al.*, 2004).

2.4 Thermodynamic approach

The same investigators who discovered that lipid membranes exhibit phase transitions already pointed out the fact that membrane transport processes can be related to the phase state of the lipids constituting the membrane (*Steim et al.*, 1969). Papahadjopoulos in (*Papahadjopoulos et al.*, 1972, 1973) detects permeation of ^{22}Na through synthetic DPPG membranes and suggests that membrane permeability changes are particularly pronounced in melting transition regime, which might be related to lipid domain formation and defects at domain interfaces. Similar observations are also made by *Petkau and Chelack* (1972). By studying osmotic behaviour of phospatidylcholine liposomes *Blok et al.* (1976) conclude that the rate of water permeation through the liposomal membranes changes drastically when passing through the phase transition temperature of phosphatidylcholine membranes. The same authors further suggested that the boundaries at the two co-existing lipid phases could be important to the formation of statistical pores in the membrane, through which the permeation of solutes would take place. Maximum permeability in the vicinity of the melting transition temperature was also noticed by *Inoue* (1974) and investigated by *Blok et al.* (1976), who also discussed the phenomenon it in terms of probability and size distribution of statistical pore formation at the boundaries of liquid and solid domains and *Papahadjopoulos* (1971) even suggests selectivity in pure lipid membranes to Na^+ and K^+ ions.

Observations of increased permeability of lipid membranes in the phase transition gave rise to developing of two permeation models for lipid membranes (*fig.*13). By some (*Tyauble et al.*, 1976; *Traeuble and Haynes*, 1971; *Cruzeiro-Hansson and Mouritsen*, 1988) the defects at domain interfaces were believed to facilitate the transmembrane transport by a simple diffusion mechanism. Enhanced domain formation and strong thermal fluctuations in the meting transition would therefore explain increased permeation of solutes across the membrane. Others (*Doniach*, 1978; *Kaufmann et al.*, 1989; *Nagle and Scott*, 1978) assumed that increased fluctuations in lipid melting transition could lead to a formation of transient hydrophilic pores in the membrane, which would account for membrane leakness under the melting conditions. Neither of the two models, however, provide detailed mechanism for the diffusion or structural information about the pores.

Thermodynamically, *Nagle and Scott* (1978) were the first to relate permeability P in the melting transition to lateral compressibility κ_T^A (and thus, fluctuations in area) and to suggest the linear relationship between them:

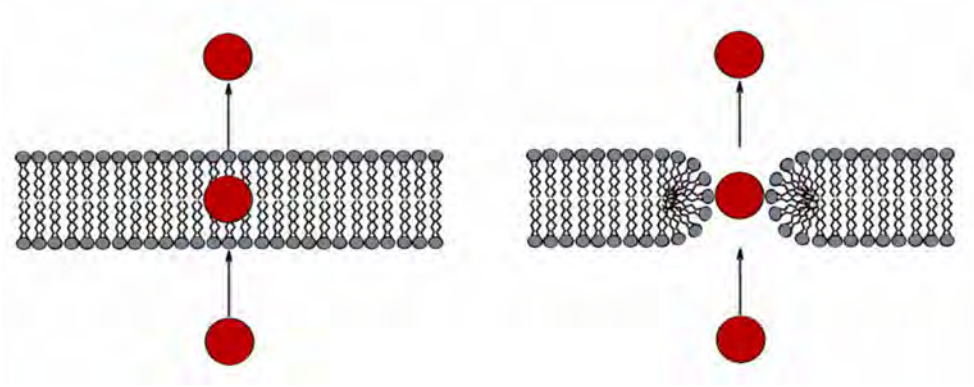


Figure 13: Two models of permeation across lipid membrane. *Left*: the solubility-diffusion mechanism in which molecules enter the hydrophobic core of the membrane due to small defects at domain interfaces and then diffuse across the bilayer. *Right*: the pore mechanism - permeation occurs through transient pores in the membrane, as a result of high thermal fluctuations. The two models provided alternative pathways of permeation of solutes through the membrane, but should not be considered mutually exclusive. Figure adapted from (*Makarov*, 2004).

$$P = c_0 + c_2 \cdot \kappa_T^A$$

c_0, c_2 - constants

Excess volume and area fluctuations, on the other hand, are proportional functions to the excess enthalpy fluctuations (*Heimburg*, 1998):

$$< \Delta V^2 > - < \Delta V >^2 = \gamma_V^2 \cdot (< \Delta H^2 > - < \triangle H >^2)$$

$$< \Delta A^2 > - < \Delta A >^2 = \gamma_A^2 \cdot (< \Delta H^2 > - < \triangle H >^2)$$

By definition, heat capacity is proportional to the fluctuations in enthalpy:

$$c_p = \frac{d<H>}{dT} = \frac{<H^2> - <H>^2}{RT^2}$$

and isothermal area and volume compressibilities can be expressed as:

$$\triangle \kappa_T^A = \frac{<A^2> - <A>^2}{<A> RT} \text{ and } \triangle \kappa_T^V = \frac{<V^2> - <V>}{<V> RT}$$

which further implies that the lateral and volume compressibilities can be expressed as linear functions of heat capacity (*Heimburg*, 1998; *Ebel et al.*, 2001):

$$\triangle \kappa_T^V = \frac{\gamma_V^2 T}{<V>} \cdot \triangle c_p \qquad \triangle \kappa_T^A = \frac{\gamma_A^2 T}{<A>} \cdot \triangle c_p$$

Finally, permeability can be expressed as a proportional function to excess heat capacity:

$$P = \alpha_0 + \alpha_2 \cdot \triangle c_p$$

which, in accordance to findings of (*Nagle and Scott*, 1978), confirms that high areas and volume compressibilities in the melting transition, where the excess heat capacity is maximum, impose the maximum membrane permeability due to high enthalpy (and thus area and volume) fluctuations in the melting regime. From this reasoning permeation via membrane pores seems to be more likely of the two hypotheses to occur, as the work which is required to create a pore against lateral pressure of the bilayer would be much smaller in the transition range, where membrane compressibility is high. This would mean that thermal motions in the membrane would increase the probability of pore formaion in lipid membrane at melting transition.

Membrane permeability in the phase transition of lipids has been a subject of research also in our lab, at the Niels Bohr Institute in Copenhagen. Andreas Blicher (*Blicher*, 2007) has shown that the maximum of the heat capacity of DPPC coincides with the maximum in permeability of rhodamine in large unilamellar DPPC vesicles (*fig.*14), while Matthias Fidorra (*Fidorra*, 2007) observed quantised transmembrane currents from DOPC:DPPC 3:1 bilayer membranes at the temperatures where heat capacity was maximum, using the BLM technique. Monte-Carlo simulations of Heiko Seeger (*Seeger*, 2006) have additionaly indicated that the the thermal fluctuations are particularly increased at domain interfaces of lipids in gel and fluid-like state (*fig.*16). Moreover, reports from (*Heimburg and Biltonen*, 1996; *Ivanova et al.*, 2003) have shown that these fluctuations are also increased in the vicinity of protein clusters in the membrane.

Temperature, however, is not the sole thermodynamic variable that can determine the melting transitions of membranes. The physics of a membrane, as of any other system, is characterised by its internal energy, which is a sum of products of intensive thermodynamic variables with differentials of extensive variables:

$$dE = TdS - pdV - \pi dA - fdl + \Psi dq + ... + \sum_i \mu_i dn_i \qquad (1)$$

p being pressure, π- lateral pressure, f- force, Ψ-electrostatic potential, μ_i - chemical potential of i- component of the system, etc.
All these contributions to the internal energy express different kind of work dW that can be applied to the system, according to the first law of thermodynamics:

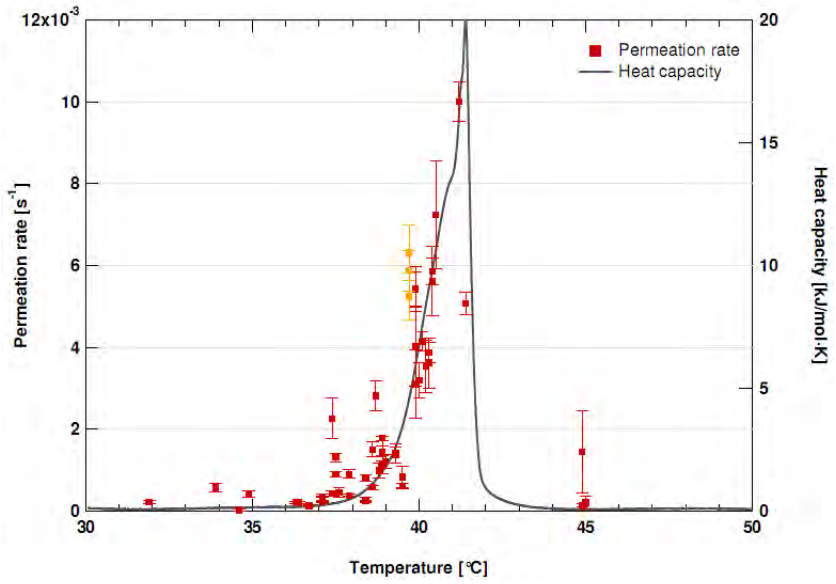


Figure 14: Permeability rate and excess heat capacity as a function of temperature for a dispersion of LUV of DPPC. Proportional dependence between permeation rate and excess heat capacity values is seen. Data collected by Andreas Blicher, by use of fluorescence correlarion spectroscopy technique (*Blicher*, 2007).

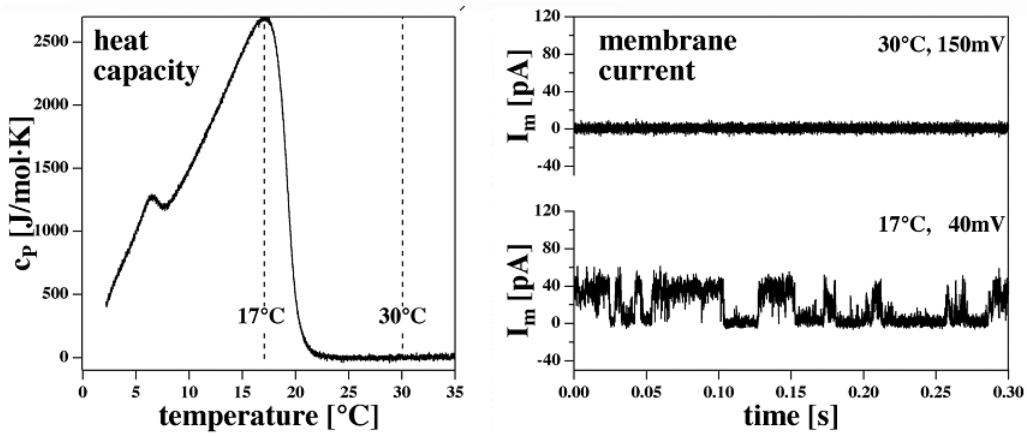


Figure 15: Descrete fluctuations in transmembrane current from DOPC:DPPC membranes at temperatures of phase transtion of the mixture. No fluctuations were seen outside the melting temperature regime. Membrane was clamped at 40mV and 150 mV with the use of BLM technique (*Fidorra*, 2007).

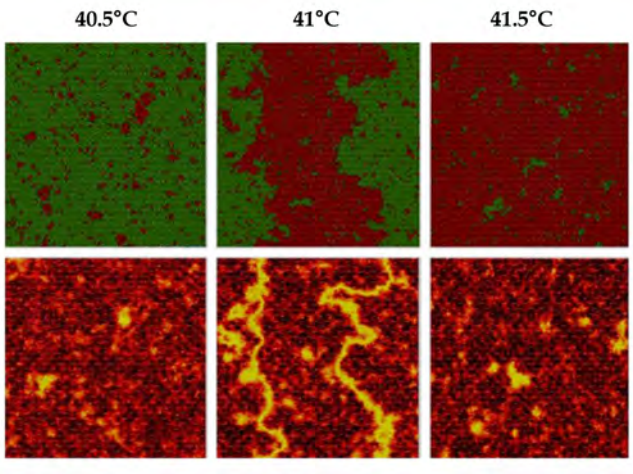


Figure 16: Snapshots from Monte-Carlo simulations of DPPC lipid membrane at temperatured in the proximity of mein malting transition ($41^{\circ}C$). *Top*: lipid domain distribution during melting transition (*red*-solid state, *green*-liquid state). *Bottom*: Local fluctuations at domain interfaces (*yellow*) are the strongest in exact melting temperature. Fluctuations are considerably smaller among domains in the same phase. Calculations are based on experimental heat capacity profiles (*Seeger*, 2006).

$$dE = dQ + dW \qquad (2)$$

where dQ denotes the change of heat of the system under consideration. The work can therefore act to change volume (i.e. of the membrane) against pressure ($-pdV$) or similarly, to change area against lateral pressure ($-\pi dA$), length ($-fdl$) or charge built-up with electrostatic potential (Ψdq).

For reversible processes, such as lipid melting phenomenon, entropy is a function of state, meaning that:

$$\oint dS_r \equiv \oint \frac{dQ}{dT} = 0$$

and according to the second law of thermodynamics:

$$dS_r + dS_i \geq \frac{dQ}{dT} \qquad (3)$$

where S_r and S_i - are reversible and irreversible components of entropy.

from which it follows that that if the system is in thermodynamic equilibrium $dS_i = 0$, any spontaneous process happening to the system will move the system away from the equilibrium state, which, in turn, will result in a value for the irreversible part of entropy S_i below the maximum.

Expressing the relation (1) in terms of entropy gives:

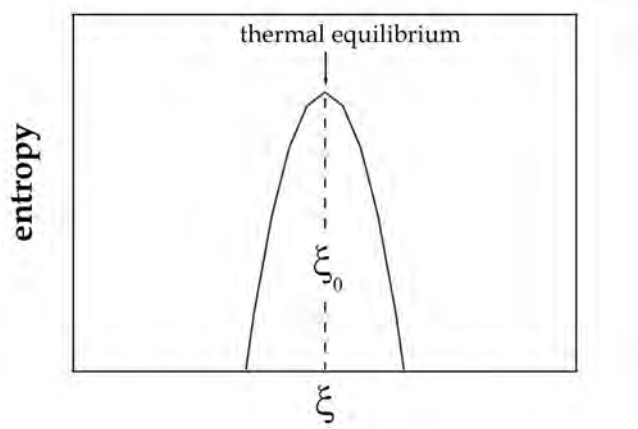


Figure 17: The shape of the entropy function depends on its irreversible component S_i, of which the maximum corresponds to thermal equilibrium state of the system, i.e. a phospholipid membrane. S_i shows approximately quadratic dependence on a reaction variable ξ. Adopted from (*Heimburg*, 2007)

$$dS = \frac{dE}{T} + \frac{p}{T}dV + \frac{\pi}{T}dA + \frac{f}{T}dl - \frac{\Psi}{T}dq - ... - \sum_i \frac{\mu_i}{T}dn_i \qquad (4)$$

which implies, that the entropy of a system, such as biological membrane, is a function of many physical variables, coupled with one another, which decide upon the thermodynamic state of the system. If $dS_i \neq 0$, fluctuations in the dependent variables will tend to restore the system back to the equilibrium state.

In more general terms, for sufficuently small fluctuations δn_i, the fluctuation strength can be quantified by expanding the entropy function to the second order in Taylor series:

$$S = S_0 + \sum_i \frac{\partial S}{\partial n_i}\delta n_i + \sum_i \sum_j \frac{1}{2}\frac{\partial^2 S}{\partial n_i \partial n_j}\delta n_i \delta n_j$$

where the strength of the fluctuations can be represented as the second order derivative of entropy $\frac{\partial^2 S}{\partial n_i \partial n_j}$and the first order derivative is the independent thermodynamic force $X_i = \frac{\partial S}{\partial n_i}$.

For a thermodynamical system with extensive variables such as energy E, volume V, area A, length l and the number of particles n_i, this force will be respectively:

$X_i = \frac{1}{T}$, $\frac{p}{T}$, $\frac{\pi}{T}$, $\frac{f}{T}$, $\frac{\Psi}{T}$, $\frac{\mu_i}{T}$ (see eq.(4))

Therefore, factors such pressure, transmembrane voltage or proton concentration (pH) can also change the thermodynamical state of the membrane, and speaking in terms of membrane melting transition and current fluctuations, they can move the system towards or away from the melting region and thus affect

membrane permeability properties. The quantitative contribution of each variable on membrane entropy cannot be assessed due to the unknown shape of the entropy function. This however, can be deduced from the shape of heat capacity profile of lipids while in the melting transition. Since membranes display a wide range of interesting physical properties at the coexistence of lipid and fluid phases, the change in these properties can be successfully studied by varying the intensive thermodynamical variables, which contribute to the entropy of the system. Pressure (*Voinova et al.*, 1990), presence of proteins, anaesthetics or pH changes have already been noted to alter the melting behaviour of lipids, which can be detected by a change in their heat capacity profiles. As it can be seen in *section 4.2* they also have significant influence on transmembrane currents and thus lipid membranes permeable properties.

2.4.1 Anaesthesia

Molecules such as ether, laughing gas, chloroform, procaine and the noble gas xenon can serve as anaesthetics. They all differ considerably in size and chemical properties but already by 1850 they had been known to posses analgesic and aneasthetic properties. At around 1900 a strong correlation between anaesthetic potency and solubility in oil had been found. This has become evident thanks to Charles Overton (1865-1933), who by investigating osmotic properties of cells observed that the permeation of molecules through membranes is related to their partition coefficient between water and oil. Hans Meyer (1853-1939) came to the very same conclusion only a few years later: the critical dose of an anaesthetics can be determined by its solubility in olive oil. The Meyer-Overton rule prompted the lipid theory, which states that general anaesthetics act through a common and non-specific mechanism by dissolving in the membrane of nerve cells, thereby causing structural changes in the lipid bilayer, in other words anesthetics_*somehow* must work on the lipid, lipophilic and/or hydrophilic sites of the membranes, displaying the same properties as in olive oil. They named the theory "lipoid theory of narcosis" (*Meyer*, 1899; *Overton*, 1901).

The word '*somehow*' has not been concretised yet. The literature offers competing theories relating anaesthetics to their effect on lipid membranes or their specific binding sites in proteins. The traditional view of the mechanisms of anaesthetisia based on the Meyer-Overton rule and general anaesthetics disrupting the lipid bilayer has been gradually discarded with the view of the latest great interest towards proteins being the main anaesthetic targets. There is a wide collection of literature on anaesthetic-induced modulation of different

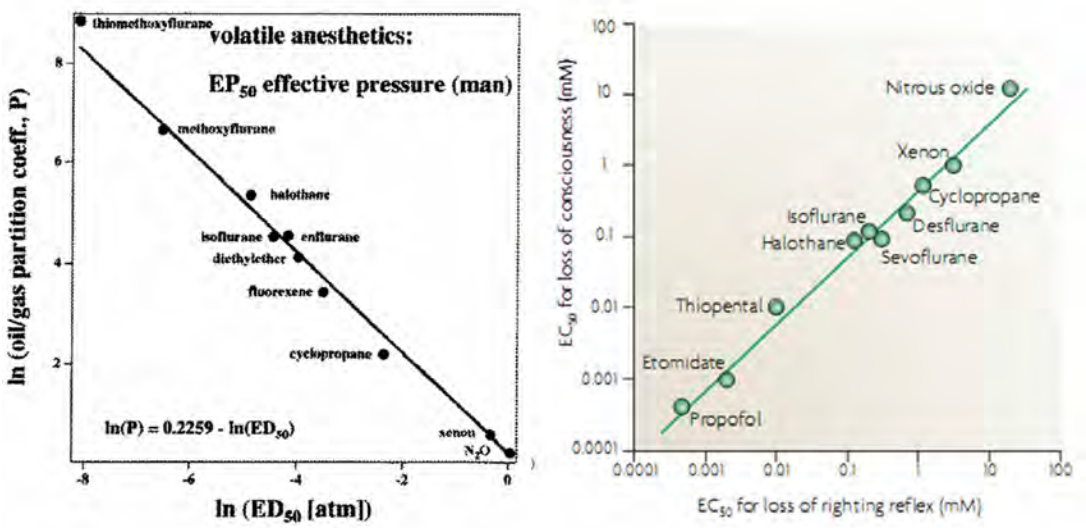


Figure 18: Confrontation with Meyer-Overton's rule. *Left*: The partition coefficient β, i.e. the ratio of the concentrations of the drug between oil and water has been found to be inversely proportional to the critical anaesthetics dose ED_{50} of anaesthetic (the concentration at which half of the population is anaesthetised)(*Overton*, 1901). Figure adopted from (*Heimburg and Jackson*, 2005). Similarly linear is the correlation between the anaesthetic concentrations that are needed to cause a loss of consciousness in humans and those that are needed to cause a loss of the righting reflex in rats and mice - *right* (*Franks*, 2008). From the definition of β it follows that at critical anaesthetic concentration the concentration of anaesthetic in the membrane is independent of the chemical nature of the drug. Even though this relation is true and commonly accepted by clinicists, the proteins are nowadays considered as main targets for anaesthesia. However, in spite of a large number of different ion channels, receptors, enzymes and other proteins that have been investigated as putative anaesthetic targets over the years now, there is strong evidence for a direct involvement in anaesthetic action for only a handful of these.

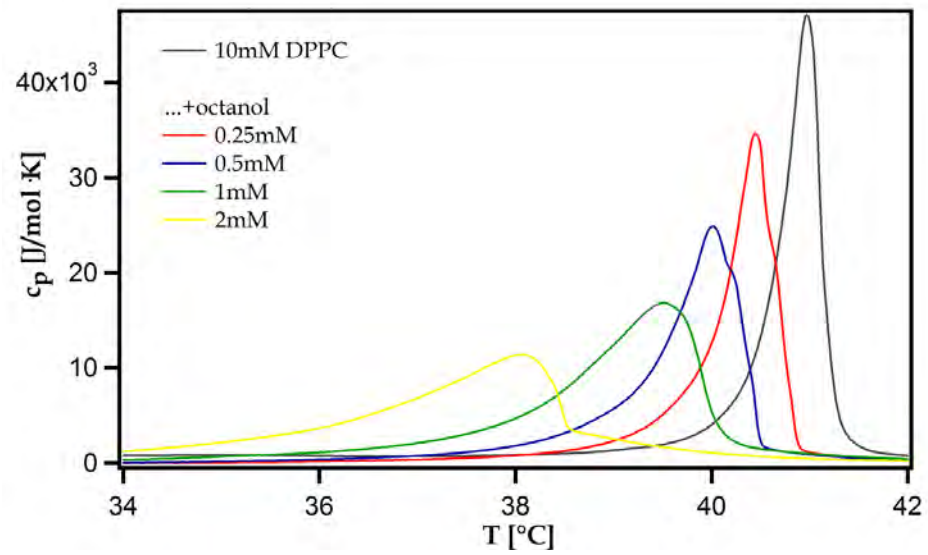


Figure 19: Anaesthetics, such as octanol, alter the melting properties of membranes (courtesy of Kaare Græsbøll, NBI, Copenhagen). The shift in melting temperature can be calculated from regular solution theory and it is found proportional to molar fraction of anaesthetics in the membrane. This can it turn be linked to critical anaesthetic dose of a drug (*Kharakoz*, 2001).

types of membrane channel proteins suggesting that they are the main sites of action of anaesthesia. Sodium ion channels were the first for which the effect of anaesthesia was observed (*Bean et al.*, 1983; *Starmer et al.*, 1984; *Grant et al.*, 1989) but there are also numerous examples of other voltage-gated ion channels being altered in the presence of anaesthetic drugs (*Josephson*, 1988).
Nevertheless, membrane properties are altered in the presence of anaesthetics too. As with any other external factor, anaesthetics change the thermodynamic state of the membrane. From the regular solution theory it can be derived that anaesthetics affect lipid melting transitions in an effect known as freezing point depression, introducing a shift in in the melting temperature point:

$$\triangle T_m = -(\frac{RT_m^2}{\triangle H})x_A$$

which is inversely proportional to melting enthalpy $\triangle H$ and proportional to anaesthetic molar fraction in the membrane x_A.
*Fig.*19 shows an example of effect of octanol on melting transition of a dispersion of LUV of DPPC. Given the proportional relation between excess heat capacity and increased permeation through lipid bilayers, the effect of anaesthesia on permeation properties of lipid membrane is intuitive and self-explanatory.

Today, it is generally accepted that there are ligand-gated ion channels, the main representatives being nicotine acetylcholine receptor (AChR), GABA

or glycine channels, which are considered the primary targets for anaesthetic molecules (*Franks and Lieb*, 1994). This is concluded on the basis of patch-clamp recordings and observations that anaesthetics modulate the current mediated by these receptors. The general mechanisms of the action of anaesthetics on protein targets is however far from being understood in detail at the moment. Different anaesthetics were shown to act on different channel types with different potency (*Peng et al.*, 2003), some of them were also shown to have contradictory effects on receptor channels even within the same family (*Deng and Chen*, 2003). Therefore, many questions in protein-mediated anaesthesia remain unanswered. In general, however, anaesthetics are known as potent inhibitors of receptor ion channel proteins, which means that in single-channel analysis they shorten the mean opening times of a channel and/or decrease the mean current amplitude of the opening state.
The nicotine acetylcholine receptor (AChR) was examined in great detail in this respect and the inhibitory action of anaesthetics on the receptor has been reported for a wide range of anaesthetics, including volatile anaesthetics (isoflurane (*Brett et al.*, 1988; *Dilger et al.*, 1993), enflurane, halothane, methoxiflurane, N_2O (*Wachtel*, 1995)), volatile anaesthetics (ether, propofol (*Dilger et al.*, 1995; *Eckenhoff*, 1996)) and some which are commonly referred to as muscle relaxants (*Colquhoun and Sheridan*, 1982). AChR function has also been studied with response to different octanol and ethanol concentrations. Therefore, the experiments performed with the Black Lipid Membranes, which also involved the application of these alcohols were put in the light of the findings on AChR channels (*section 4.4*). It is worth adding that, unlike for local anaesthetics, influence of alcohols cannot yet be rationalised with the presence of alcohol binding sites on the protein ('alcohol pockets'), which also might explain the ambiguity in findings on ethanol effect on single-channel parameters.

2.4.2 Protonic membrane transitions

The change of lipid melting transitions by changes of pH of the system is a simple consequence of the second law of thermodynamics (eq.2). While all living organisms are able to retain their body temperature at an approximately constant level, local pH changes throughout the body can undergo drastic changes, from a pH above 8 in the exocrine secretion of the pancreas, to a pH close to 0.7 in gastric acid. These, as well as protein binding to charged lipid membranes, change the electrostatics of the membrane and consequently also alter membrane melting behaviour.
Tyauble et al. (1976) have for instance shown that melting profiles of charged lipids can be changed with a different pH values of external solution (*fig.*20).

(*Kaufmann and Silman*, b, 1983) have shown that the lipid phase transition can be induced by acidification of one side of lipid membrane, and, consequently the discrete transmembrane current fluctuations would indicate an increased membrane conductivity under low pH conditions. They refer to the process as '*protonic membrane transition*', to distinguish from lipid melting transition, even though these phenomena are coupled with one another. By changing the membrane chemical potential, protons (but also proteins, anaesthetics) alter the membranes thermodynamic state, and can induce a change in the membrane lipid phase, i.e. introduce the membrane into its melting transition conditions. Interestingly enough, the protein-ion channel literature also reports many examples when proton concentration modulates the function of protein channel activity. However, insufficient understanding seems to exist in respect of this effect either. *Kwan et al.* (2006); *Trapani and Korn* (2003); *Claydon et al.* (2007), for instance, report that acidification of extracellular solution inactivates voltage-gated potassium ion channel (Kv1.5), whereas investigations of (*Cuello et al.*, 1998) seem to point toward a potentially novel gating mechanism for another type of potassium ion channel (SKC1), which is activated at low pH values. Very recently, aspirin, which is a commonly used drug for its anti-inflammatory analgesic action, has been shown to act on sensory nociceptive neurons, which bear a major category of ion channels sensitive to extracellular pH changes. They were started to be called ASIC ion channels, for acid-sensing ion channels (*Voilley*, 2004).

All these facts and together with the earlier findings in the field, have become a driving force to design experiments with BLMs and try to see whether the pH has an effect on current fluctuations seen in BLM lipids transition temperatures. Some preliminary findings in this respect are presented in *section 4.5*.

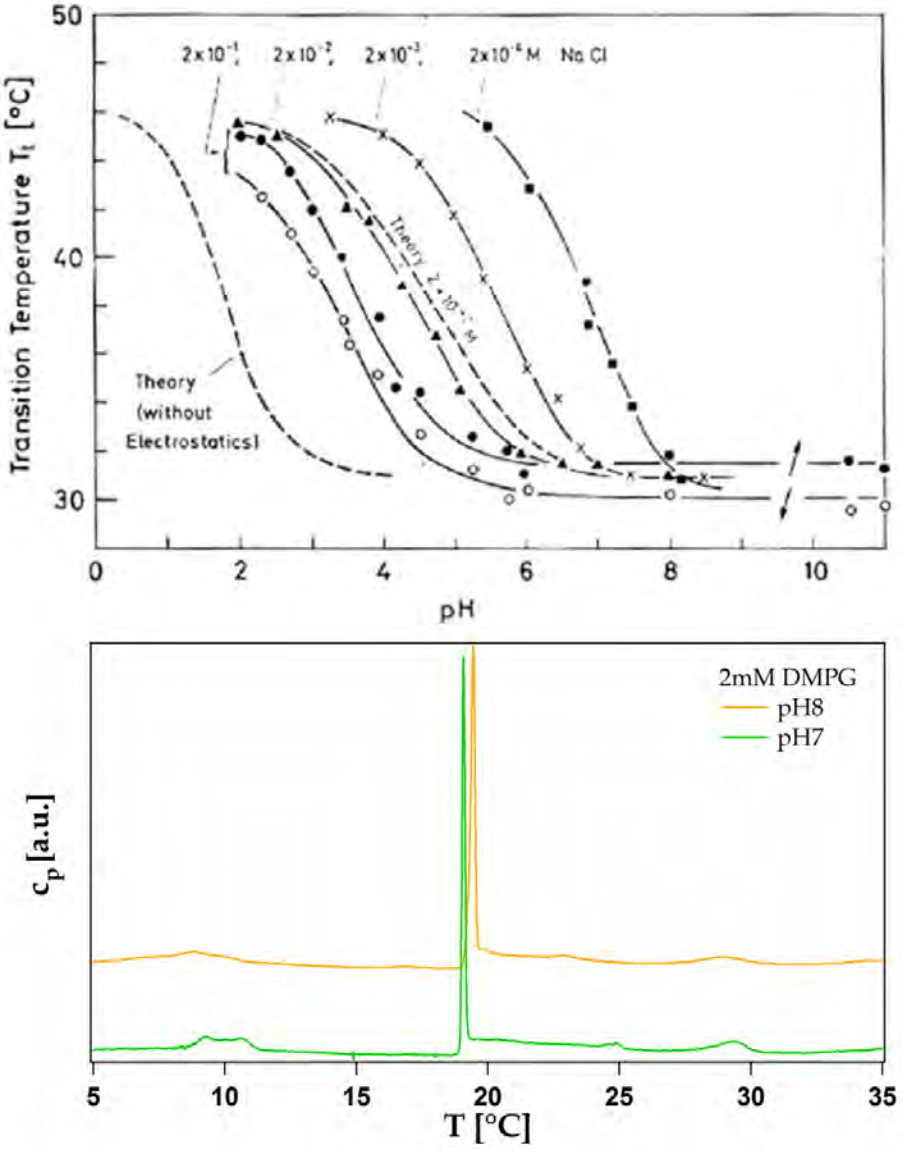


Figure 20: *Top*: Titration of methylphosphatidic acid (MPA) membranes with protons at different ionic strengths (*auble et al.*, 1976) showed that at a given ionic strength lowering the pH results in a increase in melting temperatures of lipids. The lower ionic strength moreover shifts the titration profile to higher pH values. Preliminary DSC experiments on DMPG lipid in different pHs (*bottom*) showed that pH does have an influence on melting behaviour of charged lipids (data recorded by Louise Winther, NBI).

3 Materials and Methods

3.1 Materials

Lipids: 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC), 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC) and 1,2-Dilauroyl-*sn*-Glycero-3-Phospho choline (DLPC) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) and 1,2-Diphytanoyl-sn-glycero-3-Phosphocholine (DPhPC) were obtained from Sigma-Aldrich (Germany). Lipid stock solutions were stored at -18°C, when not used in the experiments.
Decane and 1-octanol were purchased from Fluka Chemie AG (Deisenhofen, Germany). N-hexadecane, chloroform and methanol were obtained from Merck (Hohenbrunn, Germany), n-pentane was provided by BDH (Poole, UK) and potassium chloride was obtained from J.T. Baker Analyzed (Deventer, Holland). For all DSC experiments MilliQ water (18.1 MΩ) was used.

Throughout BLM experiments, the temperature was controlled by a Refrigerated Circulator Bath HAAKE DC30 K20 (Waltham, USA) and thermocouple (WSE, Thermocoax).

The picture of the teflon film with the aperture was taken with Olympus IX71 light microscope.

Conductance measurements were performed on an Axopatch200B amplifier with capacitive feedback, run in voltage clamp whole cell mode and connected to a DigiData 1200 digitizer (both Molecular Devices, Sunnyvale, CA, USA). Current traces were recorded with Clampex 9.2 software (Axon Instruments) on the hard drive of the computer with using AD converter with a time resolution of 0.1ms. The data was further analysed with Clampfit 9.2 and low-pass filtered with Bessel (8-pole) filter at a cut-off frequency of 300Hz.

For the pH-related experiments, pH-measurements were carried out with a "Sen Tix HW" pH-electrode and the pH-meter "pH 538" (WTWGmbH) of resolution 0.01. Before each measurement the instrument was calibrated with a two-point calibration with standard solutions for pH4 and pH7. Buffers used for DSC scans (Hepes, acetate, HCl-KCl) were prepared according to recipies in (*Mohan*, 2006).

Unless otherwise specified, all the figures presented in the thesis are made by Kasia Wodzinska, with the use of IGOR Pro software (`www.wavemetrics.com`).

Figure 21: The world's first ice-calorimeter, was used in 1782, by Antoine Lavoisier and Pierre-Simon Laplace, to determine the heat evolved in various chemical changes (*Lavoisier and de LaPlace*, 1780). This makes the calorimetry one of the oldest analytical methods which are in use in physics and biology nowadays.

3.2 Methods

The experimental techniques used for the purpose of this thesis comprised of Black Lipid Membrane (BLM) method, used to create protein-free lipid bilayers over a hole in a teflon film and to measure transmembrane current across them and Differential Scanning Calorimetry (DSC) as a means of determining transitions of the lipid mixtures, which the membranes were further created from.

3.2.1 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) is a powerful and very sensitive, yet easy to apply experimental technique, which uses measurements of heat changes that occur during controlled changes in temperature of a sample in a solution. By this means, it can be used to study a broad spectrum of biomolecules undergoing a transition with respect to the temperature change, including proteins, nucleic acids, lipids and detergent micellar systems. For instance, the higher the transition midpoint (T_m) for which 50% of protein molecules in a solution are unfolded, the more stable the protein is conformationally. In this respect, DSC has also been widely used to determine the change in heat capacity of protein denatuation (*Privalov et al.*, 1986).

Since the late 1960's DSC was used to study melting transitions of both artificial

and biological membrane systems (*Steim et al.*, 1969; *Reinert and Steim*, 1970; *Mabrey and Sturtevant*, 1976). More recently other types of calorimetry, such as pressure perturbation calorimetry (PPC), isothermal titration calorimetry (ITC) or water sorption calorimetry started to be used, further extending the applications of this simple thermodynamic technique (*Heerklotz*, 2004; *Plotnikov et al.*, 1997). Lipid melting transitions have been also one of the main subjects of interest in our lab. Melting transitions of pure lipid systems themselves turned out to be highly dependent on many physical variables, which clearly reflects the entropy dependence of many external variables and at the same time explains thermodynamic behaviour of assemblies of molecules, according to the second law of thermodynamics. The influence of some of them (voltage, pH change, the presence of anaesthetics) on heat capacity profile of the lipid systems studied with respect to the phenomenon of lipid pore formation is also investigated in this thesis.

All in all, DSC approaches by simply measuring heat flow through the sample, provides us with very important thermodynamic insight of the process in the sample (fusion, melting, crystallisation). In an endothermic process heat is absorbed, as happens in the case of lipid transition between gel and liquid states, whereas in an exothermic process the heat is released to the environment. In either case, electrical heating power is detected by DSC at the transition as a function of temperature. Since the difference in heating power is proportional to the excess heat capacity:

$$\triangle Q = \int_t^{t+\triangle t} \triangle P dt \approx \triangle P \triangle t$$

and the heat capacity, by definition, is the heat to increase the temperature of a system by $\triangle T$ at constant pressure:

$$\triangle c_p = (\frac{\triangle Q}{\triangle T})_p$$

one obtains direct measurement of the heat capacity change of a sample at the transition by measuring the excess heat power $\triangle P$ at a certain scan rate $^{\triangle T}/_{\triangle t}$:

$$\triangle c_p = \frac{\triangle P}{^{\triangle T}/_{\triangle t}}$$

Changes of enthalpy and entropy of the sample at the transition can be likewise determined:

$$\triangle H = \int_T^{\triangle T+T} c_p dT \qquad \triangle S = \int_T^{\triangle T+T} \frac{c_p}{T} dT$$

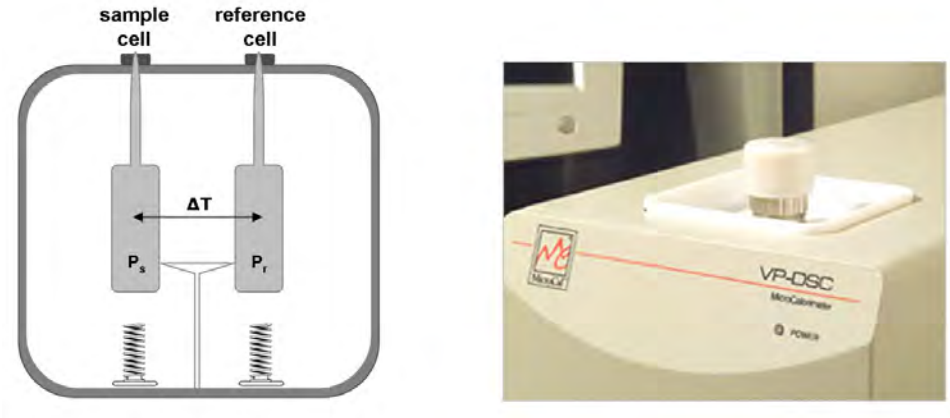


Figure 22: Schematic drawing of DSC calorimeter. The two cells are protected from the outer environment through an adiabatic shield and they are heated with a Peltier device at a constant rate so that the difference in temperature between them $\triangle T$ is kept zero. During the scan the difference in electrical heating power between the sample and the reference cell $(P_s - P_r)$ is monitored as a function of temperature.

By simply setting the start and final temperature of the scan, defining the scan rate with few other parameters like feedback mode and integration time, one is able to measure a lot of important properties of thermodynamic systems, lipid membranes being an example.
The DSC calorimeter comprises of two cells fabricated from Tantalum, enclosed by an adiabatic shield in order to prevent the uncontrolled heat leakage (see *fig.22*). One of the cells is filled with a reference solution, whereas the other contains the sample solution of interest. In our case, the reference and the sample cells were filled with a buffer and a lipid/buffer solution, respectively.

The temperature of the two cells is changed at a constant scan rate while the difference in temperature between the cells is kept at zero ($\triangle T = 0$). When the sample undergoes a temperature-induced transition of some sort (lipid melting, protein unfolding), an additional amount of heat will be required by the system and and the difference in power supplied to the two cells $\triangle P = P_s - P_r$ is measured as a function of temperature. The transition would therefore appear as a peak in the resulting heat capacity profile (thermogram) - see fig.23. Depending on the cooperative nature of lipid melting transitions, the transition half width can vary from $0.05K$ for multilamellar vesicles (strongly cooperative transitions) up to $\sim 1K$ for unilamellar vesicles.

The difference in melting profile of the lipids serves as a very useful indication for the change in overall entropy of the membrane. Therefore, on the basis of

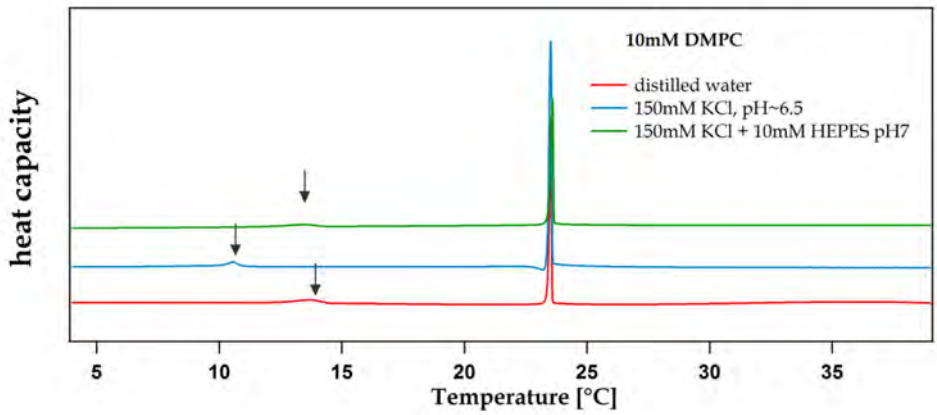


Figure 23: Heat capacity profile for DMPC lipid solutions. No difference in the heat capacity of the main transition were seen if the sample was dissolved in distilled water, 150 mM unbuffered KCl or with HEPES buffer. Only slight changes in the pretransition (arrows) could be observed (the plots were rescaled with respect to y-axis, for better clarity). More dramatic changes however are seen when, for instance, anaesthetics are present in the membrane (recall fig.19). After the baseline substraction, the excess heat capacity is left as a function of temperature, which would be further referred to as simply heat capacity change.

the shape of the heat capacity curve one can derive information on the effect of various parameters on the entropy, of which the shape is not known.

Sample preparation

For the purpose of this thesis the experiments were performed on VP-DSC calorimeter (Microcal, Northhampton/MA, USA) to measure heat capacity profiles of both single and binary lipid mixtures constituting membrane model systems in BLM experiments (see *section* 3.2.3). The calorimentric scans have been taken for the aqueous solutions of lipids alone and with the presence of anaesthetics (1-octanol, ethanol) as well as in various pH conditions.
The lipid mixture was pre-dissolved in chloroform, which was further dried under a weak flow of nitrogen gas/air and placed under vacuum overnight to remove the residual solvent. The dissolving of lipids in organic solvents, such as chloroform, before preparation of final aqueous solutions was required for homogenous mixing of the single lipid components. The dried lipid mixtures were dispersed in MiliQ water or a certain buffer to a final concentration ~30mMol/l (identical to those used in the BLM experiments). The lack of transparency of lipid samples indicates that the solution consisted of mostly aqueous multilamellar vesicle dispersions. Before filling the calorimeter, the solutions were degassed

for 10 minutes in order to remove air microbubbles. All the DSC experiments were performed at the scan rate of 5K/hr and 50-60 psi pressure (3.4-4.1 atm). High feedback mode and 4s integration time was applied.
Heat capacity profiles were analysed with IGOR Pro software (`www.wavemetrics.com`). A baseline was subtracted from the resulting thermograms.

3.2.2 Methods to study ion channels

The techniques that have been applied to study ion channels nowadays, can be classed into two main groups: the structual methods, focused on resolving the ion channels 3D atomic structure and those aiming at explaining their function and the modulation of their activity. X-ray crystallography together with light spectroscopy techniques would fall into the first category, whereas electrophysiological methods, such as the patch clamp technique or Black Lipid Membrane (BLM) method would comprise the functional techniques, simply by taking advantage of the fact that ion channels transmit a current of ions, acting as resistors in a membrane (*fig.*24).

Voltage clamp and patch clamp are the two major electrophysiological techniques widely in use nowadays in the ion channel field. Despite the fact that the work presented in this thesis employed the **BLM technique**, the experiments were performed using a professional patch-clamp amplifier. For this reason, the basic principles of patch- and voltage clamp are introduced first, before the principles of the BLM technique are explained.

Voltage and current clamp (*Hamill et al.*, 1981; *Neher and Sakmann*, 1976)

In a current clamp experiment one applies a known constant or time-varying current and measures the change in membrane potential caused by the applied current. The voltage clamp technique on the other hand involves placing the second glass electrode inside the cell in order to 'voltage clamp' the interior of the cell. Typically, in a voltage clamp experiment one controls the membrane voltage and measures transmembrane current required to maintain this voltage. The current across the membrane represents both ionic current (the rate of movement of ions across the membrane) and so-called capacitative current, which is the result of charging the capacitor-like phospholipid leaflets of the membrane (compare fig.24).

$$I_m = \frac{V_m - E_r}{R_m} + C_m \cdot \frac{dV_m}{dt}$$

The total transmembrane current can furthermore be represented by a sum of currents conveyed through the local sodium channels, potassium channels

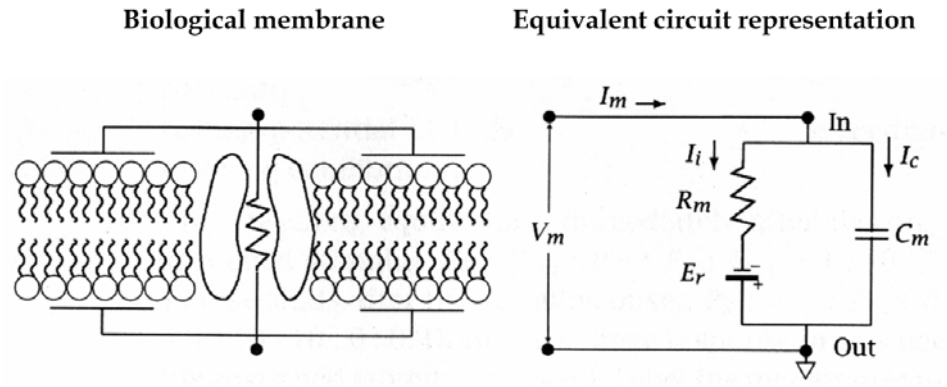


Figure 24: Membrane electrical properties compared with an electrical current. A bilayer membrane behaves electrically like a capacitor in parallel with resistors, which represent discontinuities in the membrane matrix, such as pores or channels. In this respect the phospholipid bilayer accounts for membrane capacitance, while ion channel constitutes the resistive component of the system (*HODGKIN and HUXLEY*, 1952).

etc,, and "leakage" channels, according to the Hudgkin-Huxley model of action potential (*HODGKIN and HUXLEY*, 1952).

It was not until voltage clamping was invented that a quantitative measurement of ionic currents was at all possible.

Patch clamp

Invention of patch clamp in the late 1970's (*Sakmann*, 1992) allowed the kinetics of the ion channel activity in an isolated patch of cell membrane to be measured. Unlike the voltage clamp, patch clamp uses a single electrode both to control membrane potential and to measure transmembrane current. It is so far the most prominent and widely used technique to study single protein ion channel activity (fig.25).

Recent crystallographic studies have so far revealed the structure of at least one example of a transport protein for each of the predominant ions: K^+, Na^+, Ca^{+2}, Cl^-, let alone the fact that Roderick MacKinnon was awarded a Nobel Prize in Chemistry in 2003 for the potassium ion channel structure determination. Protein ion channel structure determination is helpful when trying to examine its function in the membrane and vice versa. The function and structure must also be consistent with each other. However, the natural limitation of X-ray crystallography approach allows us to resolve the structure of a protein

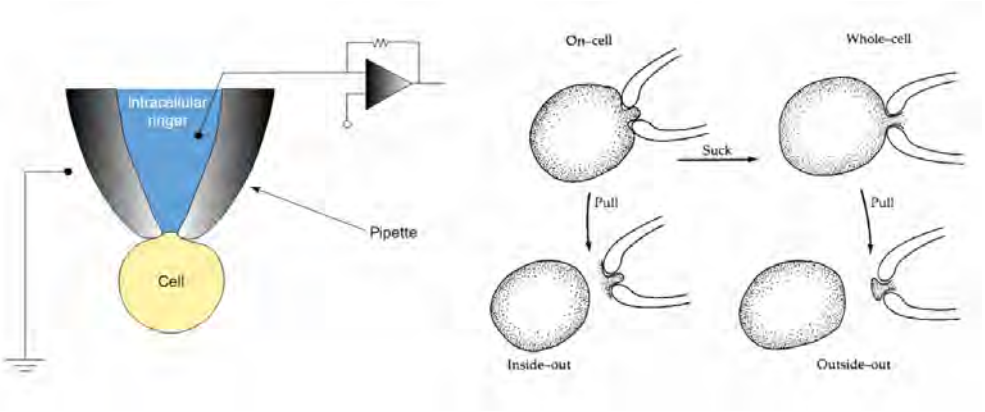


Figure 25: *Left:* Patch clamp technique (*Wood et al.*, 2004) and the main recording configurations in which it can be applied (*right*) (*Hille*, 2001). The experiments on protein-free lipid membranes, the results of which are presented in this thesis, were performed in the whole-cell mode, i.e. electrical activity of the whole membrane was measured.

under no other conditions but in its crystallised form. Therefore, with all dynamics left aside resolved structures of ion channels cannot be utterly trusted with resprct to their function. Some complementary methods must be applied to measure the structure and function of the ion channels unambiguously. The fact that proteins considered as ion channels are embedded in the biological membrane raises a fundamental question whether the protein functions are exclusively their intristic properties or are also membrane lipid-related. Findings have been already reported on differences in protein ion channel behaviour depending on the composition of the membranes they are in (*Turnheim et al.*, 1999), and their thermodynamic state (*Cannon et al.*, 2003).

All in all, many of the approaches to study protein ion channels are still in development and need to be improved in order to interpret the data correctly.

3.2.3 BLM - Planar Lipid Bilayer experiments

As mentioned above, the Black Lipid Membrane tecgnique (BLM) is, after patch-clamp technique, the most frequently used method in electrophysiology. It is based on the formation a lipid bilayer by the spreading of a dilute solution of lipids in hydrocarbon solvent over a hole in a piece of teflon foil, according to the method introduced first by Montal and Mueller in 1972 (*Montal and Mueller*, 1972). If a potential is applied across the membrane by two electrodes, the current, i.e. the rate of transport of ions, could be measured, bearing in mind the electrical representation of lipid membrane (*fig.*24). The transmembrane potential can be moreover created chemically, by introducing an ionic

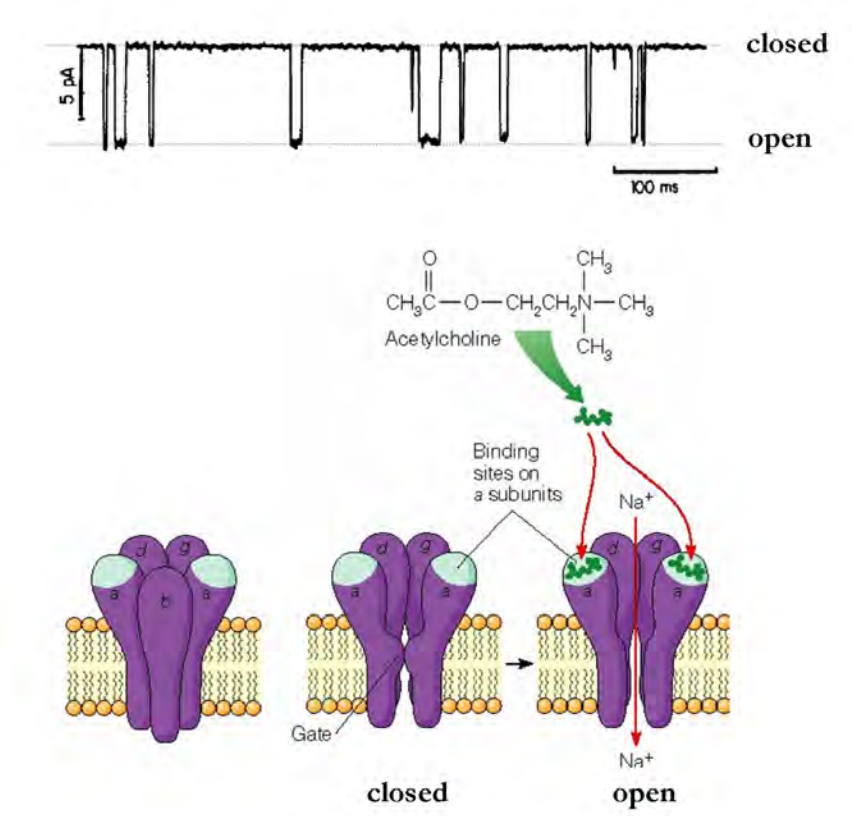


Figure 26: Ion channel opening and closing yields a rectangular current response in patch clamp/BLM techniques. *Top*: Current recording of open-shut gating of a acetylcholine receptor (AChR) channel when exposed to 300nM acetylcholine. The membrane was clamped at -140mV membrane potential and a temperature of $23°C$. From the mean current passing the channel an average flow of $4.1 \cdot 10^7$ ions/s was estimated (*Sanchez et al.*, 1986). *Bottom*: Model of a ligand-gated receptor mechanism for AChR. The channel is normally closed, but binding two molecules of acetylcholine opens the channel and lets the sodium ions through. Picture adapted from (*Darnell et al.*, 1999).

gradient in the cell and applying asymmetric solutions of ions on both sides of the membrane.

The possibility of controlling the compounds on both sides of the membrane and manipulation of lipid composition gives BLM an advantage over the conventional patch-clamp technique. Planar Lipid Bilayer experiments allow to study rare or challenging channels, which might be inacccessible for conventional patch-clamp methods. On the other hand, BLM does not allow the study of ion channels in their native surrounding, unlike patch-clamp, and due to the fact that the dimension of the teflon holes are bigger than the diameters of excised patches, the capacitances which one needs to deal with are fairly large. In BLM one also deals with slow voltage response due to resistor-capacitor filtering effect.
Nevertheless, the BLM membranes, since their inception in 1972 have been successfully used to study the structure and function of biomembranes including permeation, molecular recognition, ion selectivity and specificity, energy transduction or to detect antigen-antibody reactions (*Tien and Ottova*, 2003; *Mountz and Tien*, 1978). Furthermore, often BLM alternatives are being attached to solid supports and used as biosensors and various molecular devices. Other techniques than that proposed by Montal-Mueller have also been used to create BLMs, such as vesicle fusion directly onto solid substrates to create lipid membrane in a horizontal plane.
For the purpose of experiments, planar bilayers were formed over a round aperture in a Teflon film of 25μm thickness, dividing two compartments of a teflon chamber embedded in a brass block. The aperture of ∼50μm radius was punctured by a needle. *Fig.*27 shows the experimental set-up used together with the microscopic picture of the teflon aperture and the schematic representation of the membrane on both sides of the teflon film, after a successful BLM formation.

Sample preparation

The lipid sample was dissolved in decane/chloroform/methanol 7:2:1 (by volume), following the BLM preparation by (*Antonov et al.*, 2005, 2003). The BLM solution was kept in the freezer at -18°C, when not used in the experiments.

The aperture in the Teflon film was pretreated (prepainted) with 5% hexadecane in pentane (marked yellow in *fig.*27). The BLMs were then painted with lipid solutions of interest in decane/chloroform/methanol 7:2:1 and formed following the method described by Montal and Mueller (*Montal and Mueller*, 1972). The preliminary experiments with lipids in hexadecane/chloroform/methanol solution were not continued due to the freezing temperature of hexadecane arround $18°C$, which could affect the cuurent recordings, especially for DOPC:DPPC,

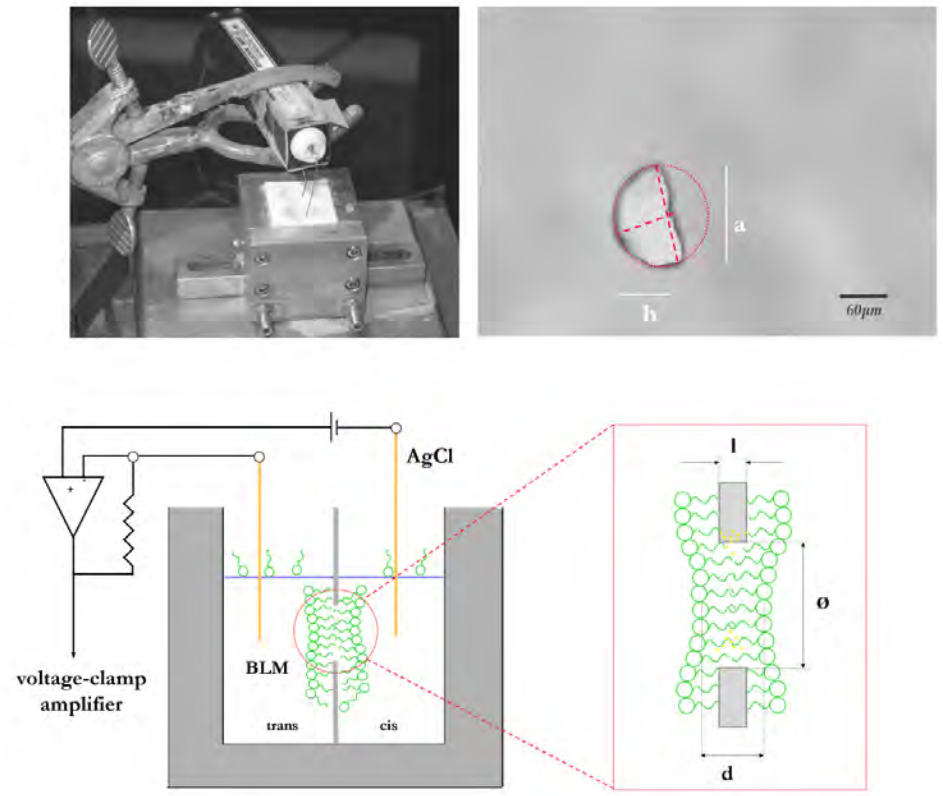


Figure 27: Experimental set-up (*top*) and the its simplified schematic representation (*bottom*). $l = 25\mu m$, $\phi \backsimeq 100\mu m$ and d represent the teflon film thickness, the aperture diameter and a membrane thickness, respectively. Scale is not preserved for the sake of visual clarity. The hole is considered as an ellipse with axes $a \backsimeq 120\mu m$ and $b \backsimeq 75\mu m$. Yellow dots in the picture of the membrane (*bottom, right*) represent the prepainting solution which was used for the purpose of minimizing the edge tension at the hole and thus to facilitate the formation of the membrane.

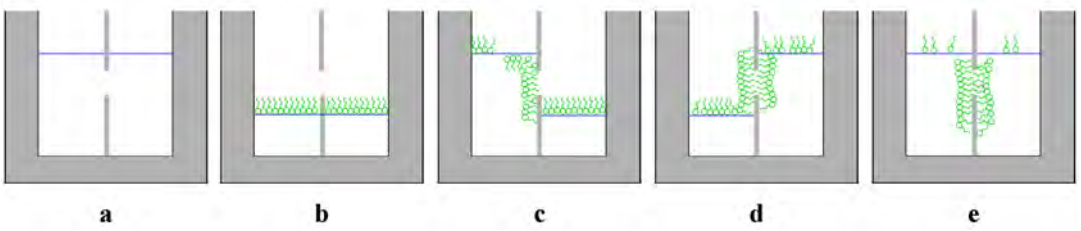


Figure 28: Formation of a bilayer membrane. Two monolayers make for the bilayer membrane across the window in a teflon film. Each stage (*a*-*e*) can be recognized by different contributions of capacitance to the overall capacitance of the membrane. This is being measured in practice by capacitance test.

for which the temperature was kept around $19°C$.

BLM formation

The two compartments of the TeflonTM block were filled with unbuffered 150mM KCl (pH∼6.5) solution and lipid solution at concentration of 25mg/ml was spread on the buffer surface in each compartment (approx. 3µl on each side). Ag/AgCl electrodes were immersed into both compartments of the chamber and a triangle voltage of 100mV/5ms was applied to the cell. After 10-15 min. time to allow for the evaporation of the solvent, the water level of the compartments was lowered and raised several times until a bilayer was formed (*fig.*28).

To qualify whether the membrane is well formed and suitable for use in the experiments both the resistance and capacitance of the membrane were measured beforehand. The built-in test in the software allowed for estimating the seal resistance of the membrane by measuring the current response to a step voltage. The seal resistance of membranes could vary in the range 1-100 $G\Omega$, where resistances smaller than 10 $G\Omega$ indicated the presence of leaks in the membrane and only larger resistances could be accepted as reasonable. The capacitance measurements on the other hand were found to be more reliable, while allowing the monitoring of membrane formation. Therefore this means of veryfying the quality of the BLM membrane was preferred thoughout the experiments.

Capacitance test

Formation of a BLMs was controlled by capacitance measurements, where calculations of membrane capacitance were performed by measuring the current response to a triangular voltage applied across the membrane. This was done in the *episodic mode* of the Clampex software.

With the constant slope of the voltage signal vs. time $\left|\frac{dV}{dt}\right| = const.$, the build-up of a charge on both sides of the membrane is linearly proportional to the voltage $Q = C \cdot V$, so that for the ideal capacitor the current response is rectangular and capacitance is linear with respect to an absolute value of current measured, as expressed by:

$$C = \frac{I}{dV/dt} \qquad (3.2.3)$$

where:
I-current amplitude
dV/dt- voltage change

The reason for non-ideal rectangular current wave is the resisitive current, which reflects the change in membrane resistance when subjected to an external field, i.e. when $dV/dt \neq 0$. With otherwise $dV/dt = 0$, the current is a typical capacitative current representing the charge build-up on the membrane, similarly when charging a capacitor. Therefore, the sloping part of current also indicates that the resistance of the membrane might fluctuate.
The conductance of the membrane was furthermore observed to be sensitive to the temperature at which the membrane was formed. The temperature-related deviations in membrane resistance are discussed in section 4.1.
Fig. *29* shows how membrane formation was controlled by detecting a transmembrane current in response to a triangular voltage wave across the membrane. No membrane present showed as infinite capacitance response (*fig.29 top*) whereas each other step of the membrane formation could be characterised by its own contribution to the overall capacitance of the membrane (*fig.29 middle*): the current contribution from the headstage was found to be of the order 0.9pA (which, taking the dielectric constant for a lipid membrane would give 0.05pF), adding ∼2ml of KCl electrolyte resulted in an increase in current to around 40pA (capacitance-wise 2pF) and when the electrolyte was in contact with the teflon film, but not in the hole itself, the charge bulid-up on both sides of the teflon film resulted in current rise to around 110 pA (to around 5pF in capacitance).

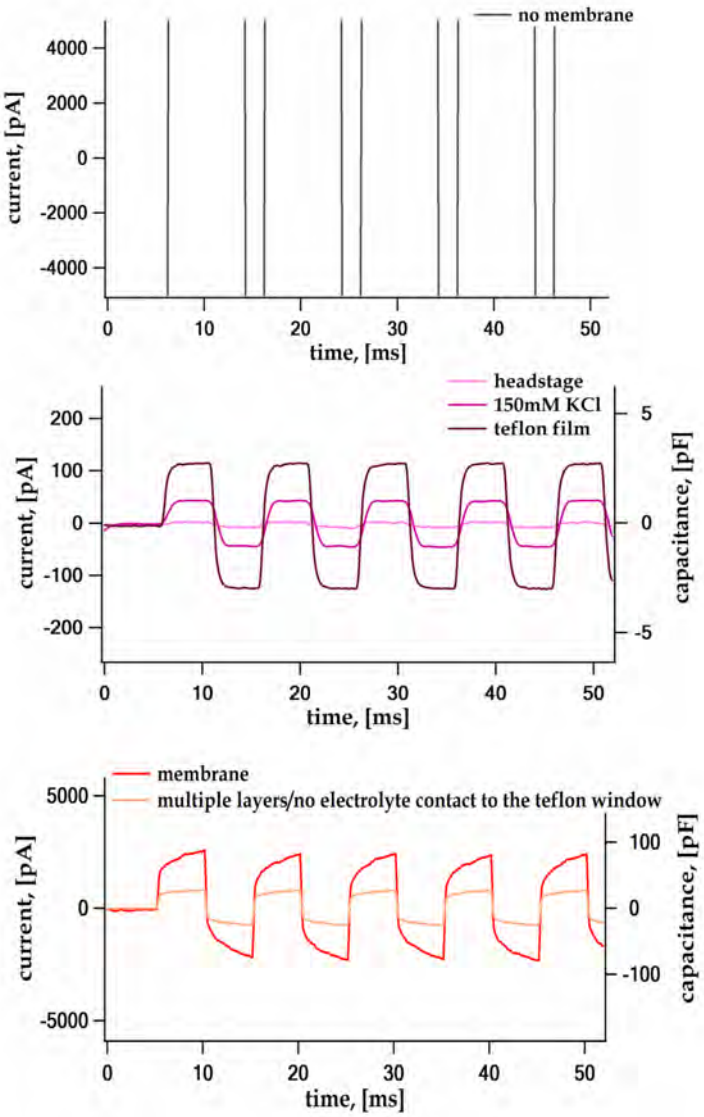


Figure 29: Current monitoring of membrane formation. Diffeent contributions to the overall membrane capacitance. *Top*: infinite current response indicating no membrane present at the beginning of the process (correspondence to *fig.28a, b*); *middle*: changes in capacitance induced by filling the chamber with the buffer to the level below the teflon film (light violet) and the case when the buffer reaches the teflon window, yet it is below the aperture (dark violet); *bottom*: membrane formation (red) - typical current response after the membrane have been formed (coincides with *fig.28d, e*); multiple layers (pink) never occured to form in a stable manner. The same capacitance pattern could also be explained by a lack of contact between the electrolyte and the teflon layer.

If the stacks of the bilayers were formed, which was rarely the case, this was indicated by a current amplitude ∼750pA (which corresponded to capacitance of the order ∼35pF), whereas the single bilayer membrane could be recognised with the current amplitude of ∼2000pA, i.e. the capacitance of the order of ∼100pF (fig.*29 bottom*).
From the readings of current in response to the triangle voltage input pulse and the calculations of membrane capacitance C membrane thickness d could be futher estimated from eq.3.2.3 and another relation defining the electrical capacitance:

$$C = \varepsilon_0 \varepsilon_r \frac{A}{d}$$

where ε_0-vacuum permittivity, $\varepsilon_0 = 8.85 \cdot 10^{-12} \frac{F}{m}$
ε_r-dielectric constant for a lipid membrane; $\varepsilon_r = 2$ (usually value from 2 to 7 is accepted (*Tien and Ottova*, 2003))
A denotes membrane area, which can be calculated as an area of an ellipse $A = \pi ab$ with $a \backsimeq 120\mu m$ and $b \backsimeq 75\mu m$ (see *fig.* 27)

*Fig.*30 depicts the practical details of the capacitance test which was done each time before the experiment. The applied triangle function is not scaled before the read-out on the screen (output scale factor $\beta = 1$) and it is equal to ${}^{dV}/_{dt} = {}^{110mV}/_{5ms}$. The current output square wave is ±2000 pA, since the gain on the amplifier is also $\alpha = 1^{pA}/_{mV}$. Recalling the dimensions of the teflon aperture (*fig.*27), the membrane capacitance of ∽100 pF is observed (the capacitance contributions of the buffer etc. are not taken into account). Following the equation relating membrane capacitance and thickness, the thickness of the membrane is calculated to be $d \backsimeq 3nm$.

In most of the cases, overall capacitance of the membrane was found to be in the order of 80-100pF, from which it follows that the membrane thickness was around 3-3.5nm. This corresponds to the membranes which were formed in the temperatures close to their melting temperatures, which is assumed to have an influence on their thicknesses as well (see*section*4.1).
With the average dimensions of the teflon aperture $\varnothing \backsimeq 100\mu m$ specific capacitance of membranes was found to be in the range ∼0.7-1.1 μF/cm^2. Capacitance per unit area of $1^{\mu F}/_{cm^2}$ is usually considered as an indication for a well-formed membrane (*Tien and Ottova*, 2003). The values ∼0.7-1.1 μF/cm^2 could therefore indicate good membrane quality. Only membranes with specific capacitance of that range were accepted for follow-up experiments.

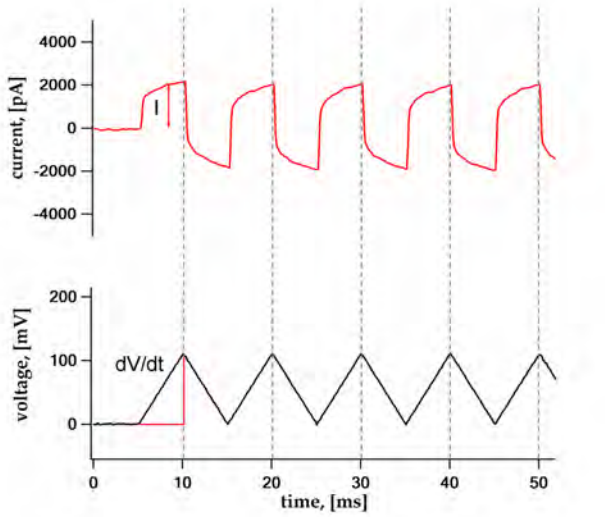


Figure 30: The capacitance test in practice. Bearing the scaling factors in mind when performing the experiments is crucial in order to calculate the capacitance values correctly. When $\frac{dV}{dt} = const.$, the sloping in the current response is seen. It is indicative of membrane conductance change.

The BLM experiments were always performed on the bilayer membranes, the stacks of more than one bilayer were very rarely observed and never found to stay stable throughout the experiments.

3.3 Single ion channel analysis

The functional information on ion channels is almost exclusively extracted from electrophysiological methods, ie. patch-clamp and BLM recordings. This is usually done through single-channel analysis, where ionic currents flowing through single channels on subconductance levels can be resolved and the dwell times of a channel in its open state can be detected.

This approach has also been employed when experimenting on lipid ion channels and the analysis has been performed in the same way that protein ion channels were first analysed using single ion-channel analysis by Neher and Sakmann (*Sakmann*, 1992).

The current traces were recorded in so-called *gap-free* mode of the Clampex software. The currents were filtered with 1kHz internal Bessel filter of the Axopatch 200B and with the Clamfit built 8-pole Bessel filter to give a final -3dB cut-off frequency of 300Hz. The signals were digitized at 10kHz sampling frequency with the sampling interval $0.1ms$.

Since so far throughout the experiments only uncharged lipids were used with equal electrolyte concentrations on both sides of the membrane, intrinsic mem-

brane potential difference was considered equal to the external electric field strength of the electrodes (with zero resting membrane potential and surface potential contributions). The electric potential gradient across the membrane φ_m could be therefore defined as:

$$\varphi_m = \frac{|\overrightarrow{E}|}{d}$$

where $\overrightarrow{E} = V_m$ - electric field between the electrodes, d - thickness of the membrane.

An activated ion channel opens and closes repeatedly until the stimulus is removed or the channel inactivates/membrane breaks due to overporation. Individual opening and closing occur randomly in an all-or-nothing fashion. The average behaviour of channel activity is characterised most commonly through analysis of the IV-curve, amplitude histograms and dwell-time analysis, from which information on mean channel conductance, current amplitude of subsequent conductance steps, mean life time of a channel and open-channel probability can be derived. The single-channel analysis favours the single-ion search process towards higher conducting channels while lower ones (less than 1pS) are usually underrepresented.
The time and amplitude analysis of single-channel recordings is based on scanning the data for opening and closing events and successfully identifying the subconductance states. With good signal-to-noise ratio the analysis is automated and provided by Clampfit software, which has also been used when analysing the ion channel-like fluctuation of lipid pores. Fig.31*top* shows the example single-ion channel recordings from DOPC:DPPC lipid membrane and the way in which the event detection was performed. The software approximates the trace to an idealised pattern, indicating the average current for each level and the mean opening (dwell) time (fig.31*bottom*).

Once current levels are identified, the characteristics of the transitions in terms of current and opening time distributions can be visualized in amplitude and dwell-time analysis histograms - (*fig.*32).
The amplitude histograms of single channel currents are made by taking the mean amplitude of the currents when the channel is open. Resulting amplitude distributions are well described by the sum of Gaussian distributions, which could indicate how many conductance levels the channels might have, for instance, a fit with a sum of two normal distributions would account for two conductance levels, whereas the separation between the peaks would correspond to

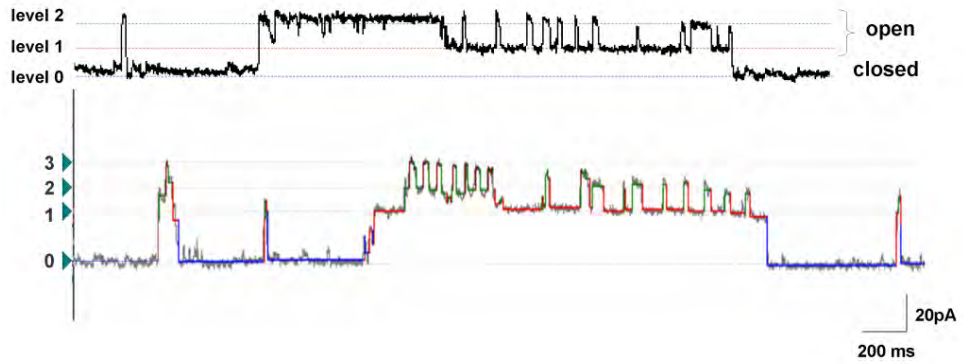


Figure 31: Defining multiconductance levels for lipid pores (*top*) together with the snaphot of the event detection performed by the software, using thresholds (0-3) (*bottom*). Level 0 corresponds to membrane 'resting state', i.e. where no pores are present in the membrane. The higher conductance levels represent the existence of pores and sub-pores in the membrane. For protein ion channels both inactivated and resting channels are nonconducting, with the difference that the first ones must be re-primed first by removing the stimulus and recover to the resting state before they get activated by an appropriate stimulus. This explanation, however, does not apply to lipid channels where the process of channel 'activation' is stochastic, i.e. nonconducting ('inactivated') membrane is simply pore-free. Both traces represent current fluctuations in DOPC:DPPC 2:1 membrane recorded at 60mV in the melting transition regime ($\sim 19°C$).

the amplitude of single channel contribution (*Colquhoun and Sigworth*, 1995). The Gaussian function:

$$f(x) = \frac{1}{\sigma\sqrt{2\pi}} \exp(\frac{-(x-\mu)^2}{2\sigma^2})$$

where μ- denotes the mean of distribution and $\sigma^2$is the associated variance.

The channel dwell times are, on the other hand, distributed exponentially. This is a consequence of the fact that single-channel events display a Markov (or memory-less) type of behaviour. After having defined the mean current amplitudes for each conductance state, the channel lifetimes can be fitted with an exponential probability density function for each level individually and the mean value of exponential distribution $f(\tau_o) = 1/e$ would refer to the mean open time for given level (*Colquhoun and Sigworth*, 1995). In a two-state model, where channel can exist either in open (O) or closed (C) state only:

$$\mathrm{O} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \mathrm{C}$$

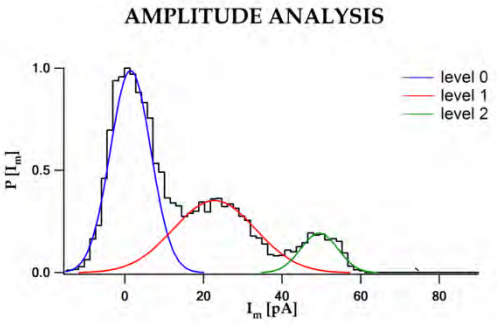
the mean duration of an individual state is equal to the reciprocal of the sum of rate constants that describe routes for leaving that state, i.e:

$$\tau_o = \frac{1}{\alpha} \text{ and } \tau_c = \frac{1}{\beta}$$

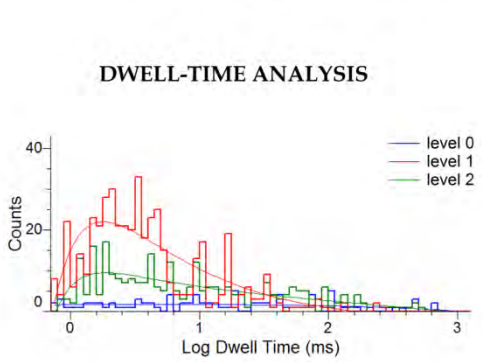
where α, β - would denote opening and closing rate constants respectively.
The exponential function used to fit the dwell distribution times was:

$$f(\tau) = \alpha \exp(-\alpha\tau)$$

Single-channel analysis and statistical analysis were always performed on complete, 30 second-long current traces.
No special analysis was employed to treat burst activity of channels.



level	P_i [%]	$\langle I_i \rangle$ [pA]	τ_i [ms]
Level 1	36.2	25.22±4.65	34.8
Level 2	22	48.51±4.73	9.88
Level 3	31.4	56.25±1.01	4.22
Level 4	10.3	60.54±2.43	4.06
		$\langle I_m \rangle$ =43.69±1.81	$\langle \tau \rangle$ =13.24



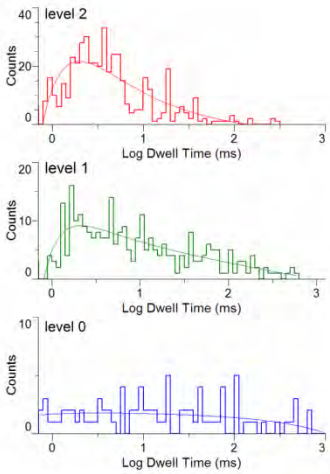


Figure 32: Amplitude and dwell-time histograms as the main outcome of the single-ion analysis. Current amplitude distribution provides us with a value of the mean current of the individual conductance levels and the their relative probabilities of occurring in the membrane: the width of prominent peak of gaussian shape around 0 deviation is a measure of high frequency background noise of current trace; multiple, equally spaced peaks at larger deviations represent possibilities that either 1, 2 or 3 channels are open simultaneously and the peak separation corresponds to the amplitude of single channel contribution. Through dwell time analysis the mean opening times of each level can be derived (*Molleman*, 2002; *Ashley*, 1996). The analysis furthermore yields the number of channels open at each level and thus probabilities of their opening at each level. The analysis relates to the sample trace from DOPC:DPPC 2:1 membrane presented in *fig.*31.

4 Results

4.1 Lipid pores - main characteristics

Throughout the BLM experiments performed, for the purpose of the thesis, symmetric solutions of 150mM KCl on both sides of the membranes were used. Having said that, experiments on the selectivity of lipid pores involving the application of an ionic concentration gradient across the membrane, the use of different ionic solutions, and membranes from charged lipids, have yet to be undertaken. The main characteristics of the lipid pores considered allow conclusions to be drawn about lipid membrane permeability under the given conditions of temperature and voltage. The analysis consists of a current-voltage relationship, estimation of the number of pores formed and the mean radius of an individual channel. Some considerations about the kinetics of pore formation and pore mean opening time are also made. Histograms of current amplitude and dwell-time, which were used to derive the main characteristics of pores, will not be shown here. The detailed analysis of amplitude and mean opening time is presented in *section* 4.2.2 which deals with influence of anaesthetics on pore formation.

Current fluctuations, possibly due to lipid pore formation in membranes, were observed for membranes composed of both singular and binary lipid mixtures at the temperatures of their melting transitions. *Fig.*33 presents the normalized heat capacity profiles for DOPC:DPPC, DMPC and DPPC lipid aqueous solutions, which were primarily used in BLM experiments and the representative current traces for each case recorded at the melting tempatures of each lipid type, under the same voltage conditions. While no prominent fluctuations were seen at temperatures away from the melting transition, i.e. in gel or fluid state, the probability of current fluctuations significantly increased when a membrane entered its melting transition.

With the use of dwell-time analysis, the mean opening time for pores in DOPC:DPPC 2:1, DMPC and DPPC membranes at 60mV were estimated as 8.2, 5.9 and 23.4 ms respectively. This, however, can only be taken as an approximation of the order of magnitude of mean open time for lipid pores, as the very narrow transitions of DMPC and DPPC made it difficult to adjust the temperature precisely and observe the current at the exact melting transition. As a result, only a few traces with current fluctuations were captured. One can, however, suppose that the mean opening time of the lipid pores are related to the lipid relaxation times, which were shown to stay in proportional relation to the heat capacity value of the transition of corresponding lipids (*Grabitz et al.*,

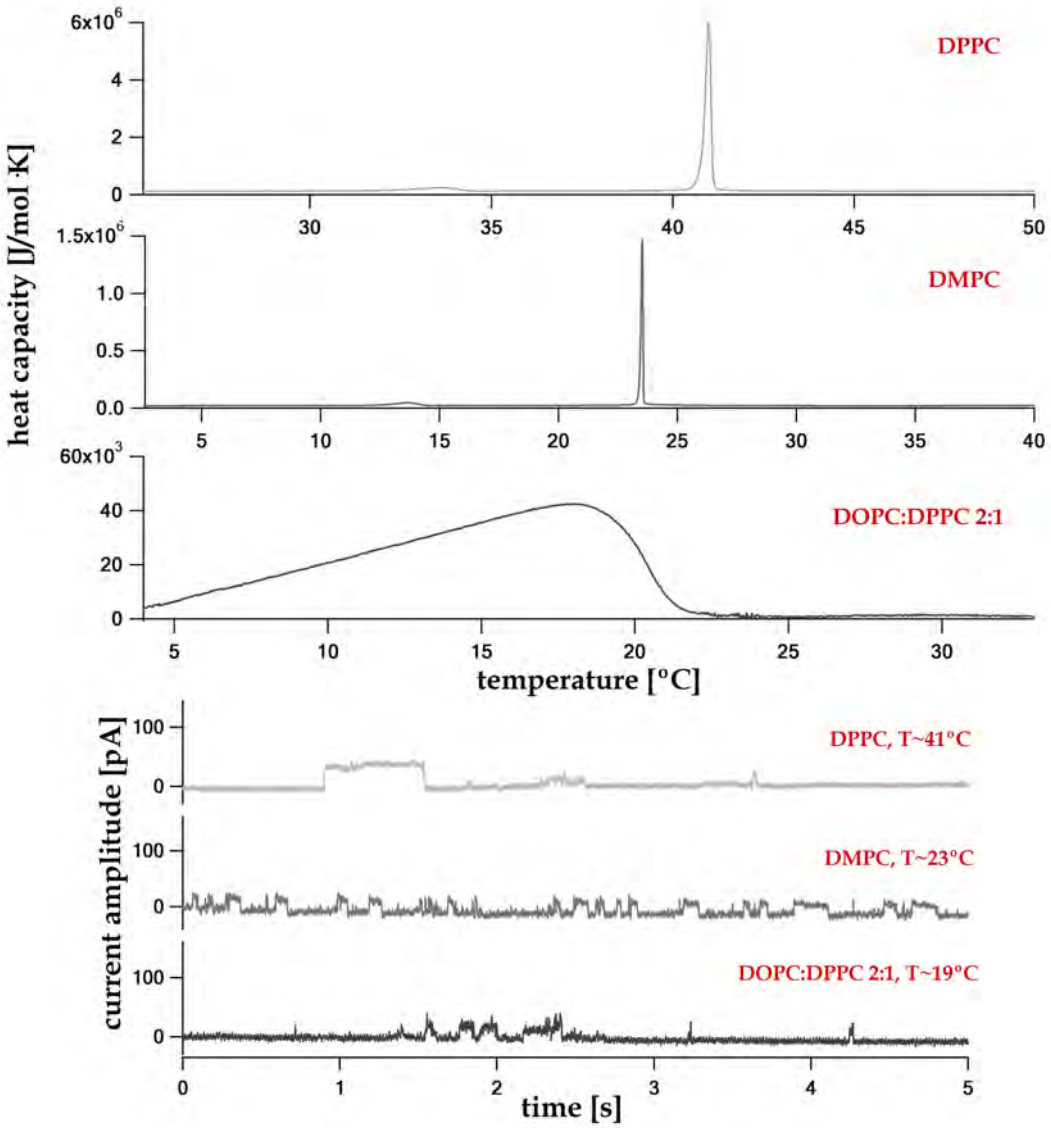


Figure 33: Discrete current fluctuations were observed for membranes made from both singular and binary lipid mixtures at temperatures in their melting transitions. Corresponding heat capacity profiles of DOPC:DPPC 2:1, DMPC and DPPC were all recorded at a sample concentration of $25mg/ml$. All the current traces were obtained at 60mV transmembrane potential.

2002; *Seeger et al.*, 2007; *Seeger*, 2006).

Different holding potentials as well as the presence of anaesthetics in the membrane also turned out to influence the mean opening time for the pores (see*section* 4.2.2).

The lipid current fluctuations can be moreover characterised by the **quantitasation** of each individual steps and the presence of **multilevel conductances**. While the first feature is also commonly observed in protein ion channel data and is not throughouly understood yet, the multiple conductance states for proteins are usually assigned to diferent receptor subtypes or different action of agonists on the single receptor subtype (the issue for acetylcholine receptor is widely discussed in literature by *Hamill and Sakmann* (1981), to give an example). Multilevel conductance steps for lipid pores can for instance be seen in *fig.34* for transmembrane potentials 120mV and higher.

Current-voltage relation

For symmetric solutions, the net flow of ions through the pore is determined by the drift of ions in the electric field between the electrodes, the transmembrane potential divided by the thickness of the membrane, i.e. to be precise. The experiments on DOPC/DPPC 2:1 membranes, which have been examined most extensively, returned an I-V relation, from which it can be seen that the membrane voltage corresponding to the zero net current through the channel is zero. This voltage, defined as reversal potential in the protein-ion channel termnology (E_{rev}), tells us that the membrane potential greater than E_{rev} would give a net positive (outward) current flow, whereas at voltages less than E_{rev} current flow is negative (inward movement of positive charge). This was indeed observed in the experiments (*fig.34*).

The I-V-plot (*fig.*34) has a linear character within approximately (-150, 150) mV range with a slight deviation from linear behaviour for higher voltages, which might indicate the membrane overporation outside this voltage range. The influence of voltage as a sole driving force on lipid pore formation is discussed in *section Appendix*.

From the slope of the linear part of IV relation the mean conductance of a pore was calculated to be $\sim 300 \pm 80pS$, which yields a value for specific channel conductance to be $\sim 3.8 \pm 0.9{}^{\mu S}/_{cm^2}$, taking the area of the whole membrane across the teflon hole into account.

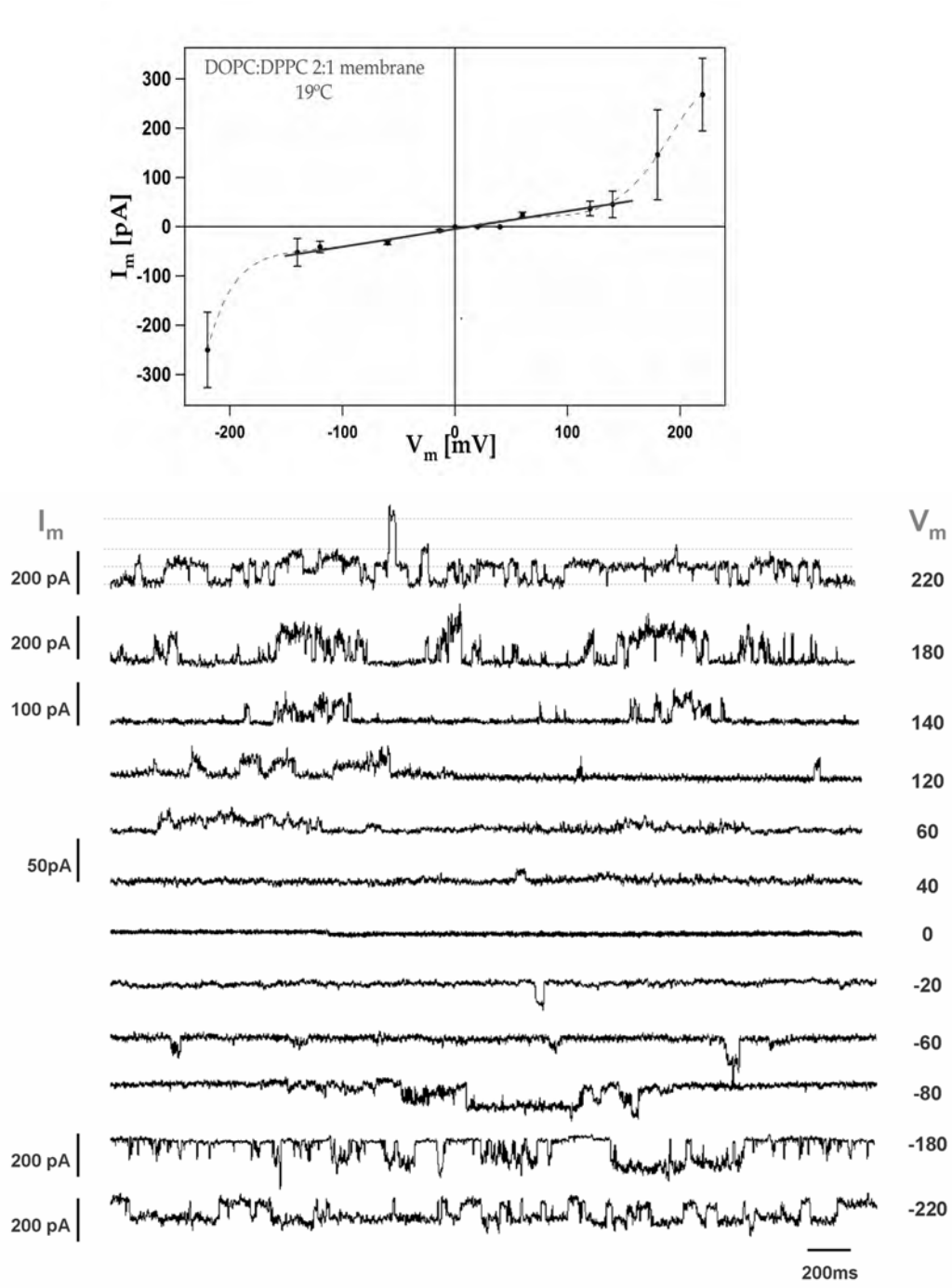


Figure 34: I-V relation for pores in membrane composed of DOPC:DPPC 2:1 mixture (*top*). The plot shows mean current ampitude for open events measured at different holding potentials. The plot is derived from an analysis of an assemble of 328 current traces (each 30s long) and calculation of average current amplitudes for each voltage, obtained from amplitude histograms. The uncertainties for current amplitude at each voltage were derived from statistical law of error propagation. *Bottom*: representative current traces from DOPC:DPPC 2:1 membrane at each holding potential.

Number of pores

Unlike a membrane with an ion channel protein, pure lipid membranes cannot be characterised with a fixed number of channels in the membrane. This is a consequence of the fact that a pore formation in the lipid membrane is not a statistical, but a random process. This also means that a closed-state of a lipid pore is undefinable, i.e. pore which has resealed is not a pore anymore but simply a lipid membrane and another pore, which appears in the membrane does it in a stochastical way. Therefore, in case of the lipid pores one could estimate a number of pores from the number of conductance levels seen in the current traces recorded, such as four conductance levels at 220mV in *fig.*34 would account for four pores in the DOPC:DPPC membrane (see also *fig.* 31).
If the representantive current traces in*fig.*34 at voltages of 60, 120, 140 and 220 mV were subjected to single-channel analysis (see *section 1.3*), which is based on an assumption that an analysed membrane does contain a protein ion channel, the analysis would yield that an overall number of pores in the DOPC:DPPC membrane increases dramatically with increased transmembrane voltage, and it varies from approximately 200 pores at 60 mV, to 500 at 120mV, 800 at 140mV and around 6000 pores at 220mV. The *fig.*35 shows the increase in the number of pores at each conductance level for the given voltages, if the single-ion channel analysis was applicable in the lipid pore number estimation.

Pore size estimation

Pore size is another parameter which is important in understanding the mechanisms of pore formation in a lipid membrane. For instance, it might be asked whether the increase in membrane current in response to the increase in voltage could also be due to an increase in individual pore size, in addition to the actual number of lipid pores (conductance levels).
Taking a pore as a cylindrical tube filled with electrolyte solution of ohmic resistance, the pore radius R_{pi} can be estimated from the average single pore conductance G_{pi} as:

$$R_{pi}^2 = \frac{G_{pi} \cdot d}{\pi S}$$

where d is the mean thickness of the BLM bilayer and S is the specific conductivity of the electrolyte solution surroundung the membrane (*Antonov et al.*, 2005). The latter follows from Kohlrausch's Law, which states that the molar conductivity of a solution changes as a function of the square root of the concentration of the solution (*Hallock*, 1900). The specific conductivity of 150 mM

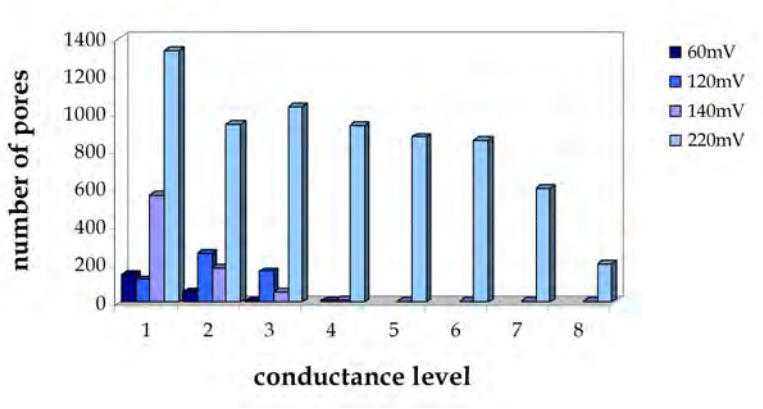


Figure 35: Provided the membrane is not protein-ion channel-free, the single-ion channel analysis would yield a mean number of channels in the membrane for each value of transmembrane voltage and with respect to the individual conductance steps. The result would appear as an increase in the number of lipid pores with voltage. The higher the potential across the membrane, the more conductance levels could be also seen (ranging from 2 at 60mV up to 8 at 220 mV) and a decrease in the number of pores at higher conductance levels compared to the number of pores at lower conductance levels could be observed. Analysis included 30s-long current traces, representative for each voltage for pores in for DOPC:DPPC 2:1 membrane. The number of pores are given per whole area of the clamped membrane.

KCl, which was used as a membrane solution in all the experiments, was found to be equal to $7.5^{mS}/_{cm}$.

With the average membrane thickness throughout the experiments estimated to be $d = 3.2 \pm 0.1nm$, from the mean current amplitude $\sim 24.6 \pm 4.9pA$ at a transmembrane potential of 60mV (see *fig.*34), the mean pore radius could be estimated as $R_{pi} \sim 0.75 \pm 0.11nm$. This value is comparable with the mean area per lipid headgroup for DPPC molecules and it is also of the same order of magnitude as the size of selectivity filters of potassiumm ion channels and acetylcholine receptor channels for sodium ions (see part in *section* 6).

It is worth mentioning however, that the above approach of deriving the mean size of a pore in a membrane is only true if the pore is filled with an aqueous solution. The properties of the solution might change significantly if the conductance of the membrane was due to the presence of ordered water at interfaces and the resultant quantised currents were a consequence of dielectric breakdown of the membrane.

Capacitance and conductance change

In BLM experiments on DPPC membranes, which required a substantial temperature rise from the room temperature ($\sim 20°C$) to bring the lipids to their

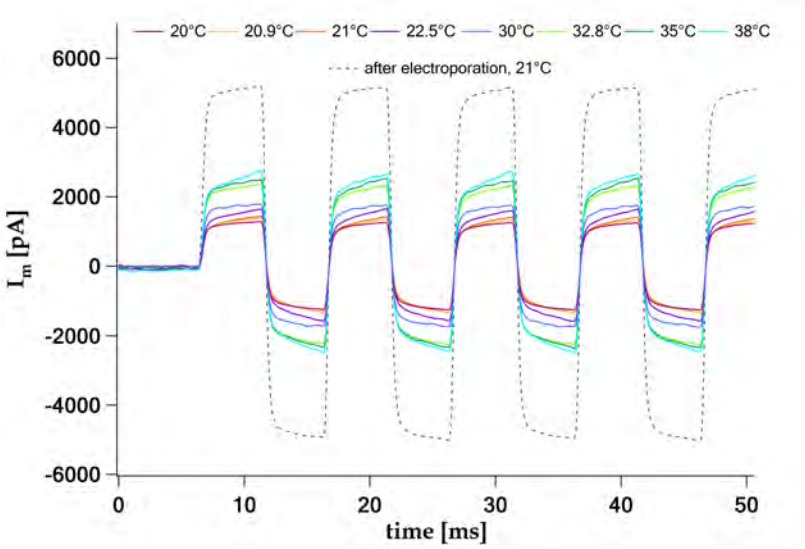


Figure 36: Capacitance and conductance changes seen with DPPC membrane at different temperatures. The closer the temperature to the melting temperature of the lipid ($\sim 41°C$), the larger membrane capacitance and more increased sloping of the current response, pointing to non-zero resistive current across the membrane. Capacitive currents were recorded for a few passages through lipid melting transition (by cooling and heating the membrane), in response to a triangular voltage signal of $110^{mV}/_{5ms}$.

melting transition ($\sim 41°C$), a change of capacitive current at different temperatures was observed (*fig.*36). This change in membrane electrical capacitance provided an indication of the change in membrane thickness at different experimental temperatures. The decrease of thickness of a DPPC bilayer in the temperature range of $\sim 20°C$ to $\sim 38°C$, averaged from a number of passes through the lipid melting transition, was measured to be of the order of 48% (*fig.*37). Earlier observations of the change of membrane thickness at the phase transition reported a changes of 17% (*Gennis*, 1988) and 65% (*Antonov et al.*, 2003).

This, however, cannot be taken as the only explaination of the observed capacitance change with the temperature, as the melting transition itself is more likely to change membrane thickness by only $\sim 20\%$ (*Heimburg and Jackson*, 2005). Therefore, the maximum capacitance at the melting transition is only partially attributed to the thinning of the bilayer and the effect is mainly believed to be caused by solvent reditribution in the membrane (*Antonov et al.*, 2003; *Bagaveev et al.*, 1981). In this respect, the change in membrane capacitance can be considered as having both geometrical component, accounting for the membrane area and thickness change at the transition (also suggested by *White* (1975)) and a

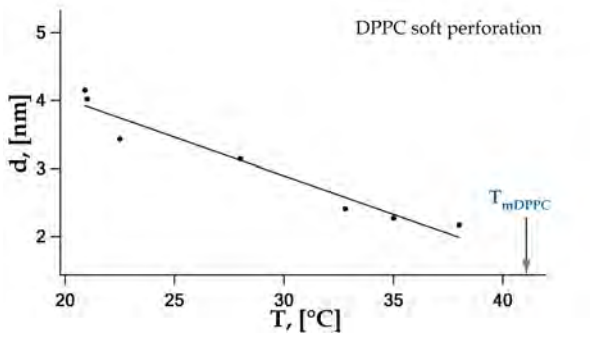


Figure 37: Estimation of reduction in membrane thickness as a function of temperature for DPPC membrane, taken that the increase of membrane capacitance is due to the membrane thinning exclusively. Nevertheless, other factors need to be taken into account to interpret the process correctly (see text). The increase in membrane capacitance at temperatures close to the main melting transition also coincides with an increased probability of pore formation in the membrane.

physical one, which can be attributed to decane changing the dielectric constant for a lipid membrane (personal discussions with V.Antonov). Furthermore, the change in the lipid headgroup reorientation in the melting phase transition could also give rise to the observable change in membrane capacitance, which can no longer be judged from the equation relating membrane capacitance with the dielectric constant and the membrane thickness (see also *section* 6).
From the increased slope of the current response in the vicinity of melting temperature in *fig.*36 it can also be concluded that the resistive current is not zero when the membrane is close to its temperature melting regime. This fact was also observed by *Antonov et al.* (2003) for BLM membranes from hydrogenated egg lethicin, which showed increased membrane ionic conductance in the vicinity of their phase transition. This further coincided with earlier observations of pore appearance in membranes at phase transition and provides complementary proof for the increased membrane permeability. An additional clue to the correlation between temperature-modulated changes in membrane capacitance and increased ionic membrane conductance can be suggested by the current response after the membrane was subjected to a very high voltage ($\sim 400mV$), at which it soon broke down - dotted line in *fig.*36. The presence of electrically induced pores in the membrane could therefore also be seen from the change in overall membrane capacitance.

Burst activity

Burst activity, i.e. the presence of current fluctuations only at certain time intervals with no activation present elsewhere was also observed frequently for lipid

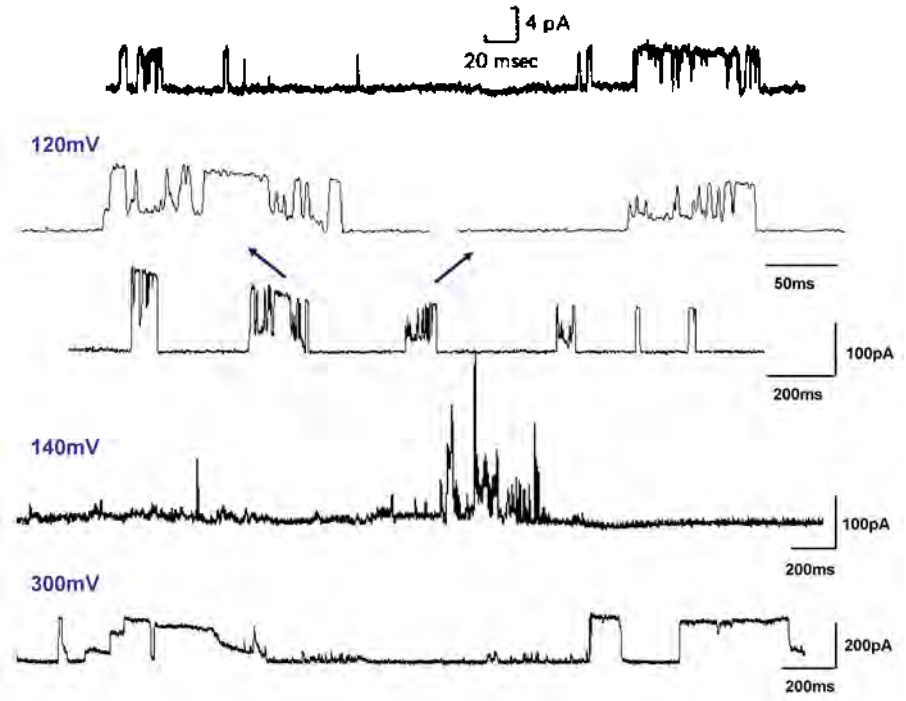


Figure 38: *Top*: Burst activity for Ca-activated potassium ion channel in a rat muscle (*Magleby and Pallotta*, 1983) and the bursts, spikes and quantitised steps observed in current traces from a DOPC:DPPC 2:1 membrane in the melting transition temperatures ($\sim 19^{\circ}C$), 150mM KCl, pH$\sim$6.5 (*bottom*).

pores. The phenomenon is known in the protein-ion channel field for some potassium and calcium ion channels and also for GABA receptor (*Puia et al.*, 1990) and acetylcholine receptor channels (*Neher and Sakmann*, 1976). *Fig.*38 shows the examples of bursts in current fluctuations accountered in DOPC:DPPC 2:1 membrane and an example of bursting activity for potassium ion channel (*Magleby and Pallotta*, 1983).

Blocking

Last, but not least lipid pore formation phenomena seem to be sensitive to the same blocking agents as protein ion channels activity, for instance octanol or ethanol (*section* 4.2.2), often referred to as general anaesthetics. The membrane permeability can be moreover controlled by a range of other physical factors, such as temperature, voltage or the pH of external solutions (see next), all of which are found to contribute to the overall entropy of the membrane (see *section 2.4*).

4.2 Lipid channel gating?

Factors contributing to membrane entropy

According to the overall expression for the internal energy of the system given in*section 2.4*, the state of a lipid membrane, as any other thermodynamic system, is determined by the sum of products of intensive variables (temperature, electrostatic potential or chemical potential of the system) and changes in extensive thermodynamic variables. The same set of variables could strongly influence the process of pore formation in the membrane and effect lipid pore characteristic features, as mean curent amplitudes and mean opening times. In protein ion channel terminology, they could influence the gating mechanism of channels.
In the following sections the factors such as temperature, transmembrane voltage and the pH change of the electrolyte will be shown to modulate the lipid pore activity in unmodified lipid membranes. The action of anaesthetics, on the example of octanol and ethanol, was found to inibit the current fluctuations in a significant way. The influence of the presence of anaesthetic compounds in the lipid membrane on the lipid melting profiles is also discussed.

4.2.1 Temperature vs. Voltage

Temperature was the first factor that was found to be crucial in determining the presence of lipid pores in the membrane by an observation of discrete current fluctuations (*Antonov et al.*, 1980, etc.).
Experiments from the 1980's confirmed the fact that the probability of observing the discrete current fluctuations across the membrane is the greatest when lipids are undergoing the phase transition (recall *fig.12*). *Fig.*39 shows the irregular fluctuations observed in the DOPC:DPPC membrane below the maximum melting temperature (*fig.*39*A,a*)and no observable fluctuations at the temperature above the melting point temperature (*fig.*39*C,c*). At $19°C$ the current fluctuations revealed quantitised character, which can be interpreted as ongoing transitions from open and closed states of the pores (*fig.*39*B,b*).

Transmembrane voltage is naturally the other factor that can modulate the lipid pore formation, which could already be seen from the I-V relation for pores in DOPC:DPPC 2:1 membrane in *fig.*34. When at the melting transition, voltage affects the mean current amplitude crossing the membrane in an approximately linear manner and excessive voltages (above around 180mV) lead to membrane overporation and non-linear current-voltage behaviour and, if maintained for too long, inevitably to membrane breakage. When away from the melting transition temperatures, the transmembrane voltage was also observed to induce the current fluctuations in membranes made from DPPC and DPhPC lipids

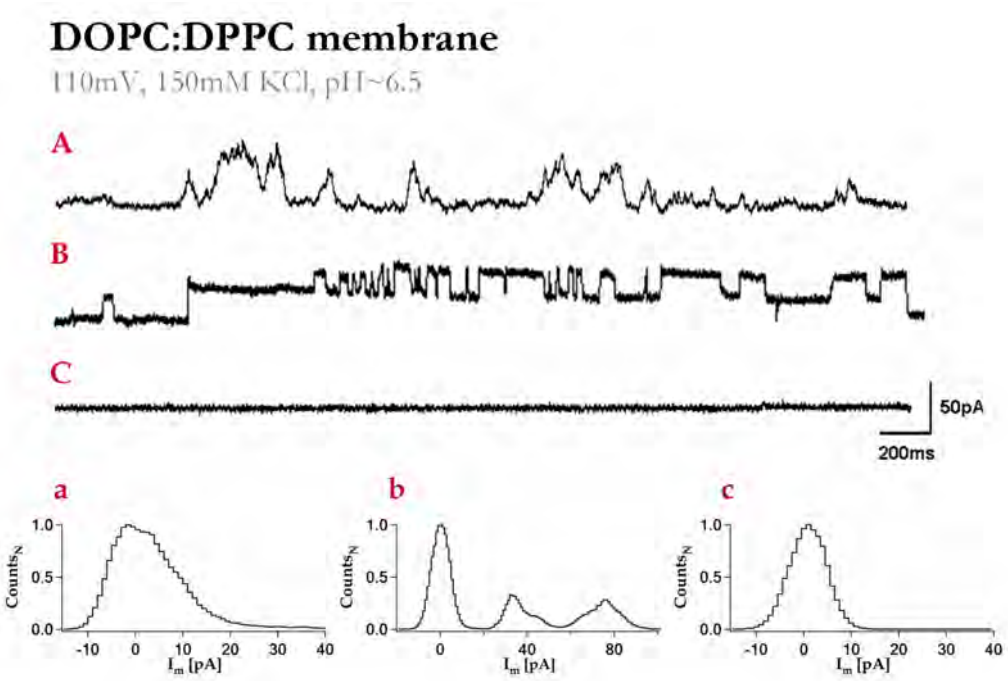


Figure 39: Current recordings from DOPC:DPPC 2:1 membranes at different temperatures with respect to the melting transition temperature and the corresponding amplitude histograms. Below the melting temperature range some non-discrete fluctuations could be seen (A), whereas at temperatures above from the transition no fluctuations were present (C). When in the melting transition (B), discrete current fluctuations were seen, which is also reflected by the current amplitude distribution (b). Amplitude histograms of currents below and above the melting temperature regime show no pronounced peaks, with the only difference being that the fluctuations below melting temperature (a) result in broader histogram profiles than those of a temperature below the transition (b). Traces A, B, C were recorded at $15°C$, $19°C$ and $23°C$, respectively, all at a constant transmembrane potential of $110mV$.

(see *section Appendix*). A plausible explaination for that happening could be the reorientation of lipid head groups in an external electric field, which could in turn account for change in the phase of the lipid molecules from gel into liquid-like (see also *section Appendix*).

4.2.2 Anaesthesia

A set of BLM experiments were performed in order to see whether general anaesthetics have an influence on lipid channel activity as they are frequently reported to do so for protein ion channels. Muscle and neuronal homologues of acetylcholine receptor channels (AChRs) are the example of receptor channels which have been extensively studied in terms of their response to different anaesthetics. They have been found to be very sensitive to many anaesthetic agents, which, taking into consideration the fact that they are widely distributed in the central and peripheral nervous system, is believed to be of great importance in understanding the modulation of these receptors by anaesthetics. Furthermore, the AChRs are also assumed to be involved in pain processing and antinociception (*Marubio et al.*, 1999), which makes investigation of the interactions with anaesthetics even more important. All in all, AChRs have become a model of channels for the effects of anaesthesia were studied and the mode of anaesthetic action was proposed. They were therefore used as a model in planning the experiments with lipid ion channels and anaesthetics and the results obtained were compared with the observed effects of anaesthetics on AChR (and other) protein ion channels.

For that purpose, the same, DOPC:DPPC 2:1 lipid mixture, was used due to the proximity of the melting temperature to the temperature at which the experiments were performed (see heat capacity profile of aqueous DOPC:DPPC 2:1 lipid mixture in *fig.33*), and thus relatively easy temperature control.

Calculations of the anaesthetics fraction in the membrane

From the definition of partition coefficient of the anaesthetic in the membrane:

$$\beta = \frac{c_m}{c_b}$$

where: $c_m = \frac{n_m}{V_m}$, $c_b = \frac{n_b}{V_b}$ and $n_t \equiv n_a = n_b + n_m$

and: β- partition coefficient of the anaesthetic under consideration

c_m, c_b - molar concentrations of anaesthetic in the membrane and buffer, respectively

n_m, n_b - number of moles of anaesthetic molecules in the membrane and in the buffer

$n_a \equiv n_t$ - total number of moles of anesthetic molecules added to the

system

V_m, V_b - membrane volume and volume of the buffer

the concentration of anaesthetic in the membrane can be derived as:

$$c_m = \frac{\beta \cdot n_a}{\beta \cdot V_m + V_b}$$

which is equivalent to:

$$n_m = \frac{\beta \cdot V_m}{\beta \cdot V_m + V_b} \cdot n_a$$

The fraction of anaesthetic in the lipid membrane in $[mol\%]$ can be derived as:

$$\tilde{c} = \frac{n_m}{n_m + n_l}$$

with - n_l being number of moles of lipids in the membrane.

For small amounts of anaesthetic partitioning the membrane $n_m \ll n_l$ and it can be assumed that:

$$\tilde{c} = \frac{n_m}{n_l}$$

Thus, the molar fraction of anaesthetic molecules in the membrane can be derived as:

$$\tilde{c} = \frac{\beta \cdot V_m}{\beta \cdot V_m + V_b} \cdot \frac{n_a}{n_l}$$

Finally, assuming that the densities of lipid membrane and the buffer are similar $\rho_m \approxeq \rho_b \approxeq 1 {}^{g}/_{cm^3}$ and the membrane was created from all the lipids used, one arrives at:

$$\widetilde{c} \approxeq \frac{\beta \cdot m_m}{\beta \cdot m_m + m_b} \cdot \frac{n_a}{n_l} = \frac{\beta \cdot m_m}{\beta \cdot m_m + m_b} \cdot \frac{m_a}{m_l} \cdot \frac{M_l}{M_a} \approxeq \frac{\beta \cdot m_a}{\beta \cdot m_m + m_b} \cdot \frac{M_l}{M_a}$$

So with $m_m \approxeq m_l$:

$$\tilde{c} = \frac{\beta \cdot m_a}{\beta \cdot m_m + m_b} \cdot \frac{M_l}{M_a}$$

where: m_a- mass of anaesthetic added to the system

m_m- mass of lipids used

m_b- mass of buffer

M_l, M_a - molar mass of lipids and anaesthetic respectively.

In the sets of experiments with octanol 2x1, 2x2, 2x3 µl of 15% v/v octanol in methanol solution was subsequently added to the system, i.e. 1 µl of octanol solution was added to each side of the membrane and it was increased by 1 µl for the following experiment.

Calculations showed that the molar fraction of octanol in the membrane in each case was:

- for 2µl total amount of octanol in the system: 7.93mol %
- for 4 µl: 15.85mol%
- for 6 µl: 23.78mol%

In the experiments with ethanol, 2x30 µl and 4x30µl 99% ethanol was added to the BLM membrane, which gave:

- for 60 µl total amount of ethanol in the system: 9.5 mol %
- for 120 µl: 19 mol %

Partition coefficients for octanol and ethanol were assumed to be equal to $\beta = 201 \pm 2$ (*Jain and Wray*, 1978) and $\beta = 0.48$ (*Firestone et al.*, 1986), respectively.

Octanol

For the experiments with octanol, 15% v/v octanol in methanol solution was prepared and added symmetrically on both sides of the membrane in the teflon chamber under defined conditions of voltage and temperature (210mV, 19°C). Due to the high octanol/lipid partition coefficient (*Jain and Wray*, 1978), the holding membrane potential was chosen to be relatively high in order to be able to quantitatively analyse the influence of the anaesthetic on the current fluctuations. It is worth mentioning however, that the experiments were also carried out with lower holding potential, with the same resulting inhibitory effect of octanol on the current fluctuations.
The experiments, of which the results are presented below, were performed on the same lipid bilayer, the thickness of which was estimated (from the capacitance measurement) to be of the order of 3.6 nm.

The inhibitory effect of octanol on current fluctuations in lipid membranes was found to be similar to the action of octanol on protein ACh receptor channels - see *fig.*41.

Amplitude and open time distribution analyses

Amplitude and dwell-time analyses were performed on the single-channel recordings from lipid pores with and without octanol in order to describe the effect of octanol on lipid pore behaviour in a more quantitative manner. The analyses have shown that both mean open time and amplitude of single currents were markedly affected by the presence of octanol in the membrane. The results were

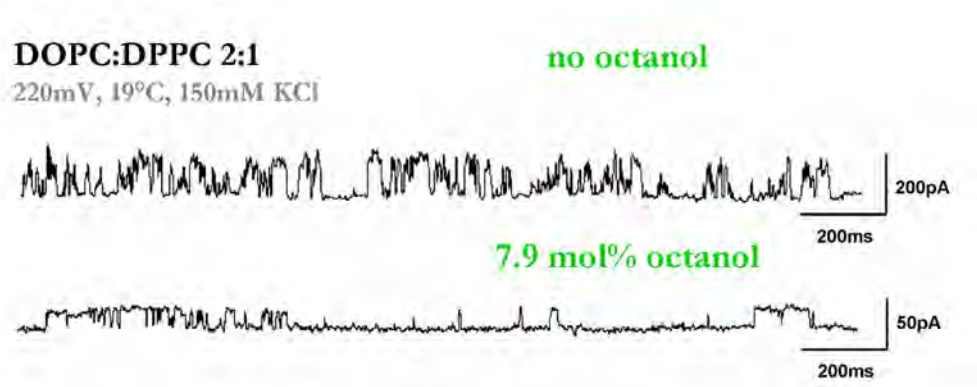


Figure 40: Inhibitory effect of octanol on the lipid pore formation. The difference in mean current amplitude of the current fluctuations in the presence of octanol in the membrane can immediately be seen. Picture presents the current fluctuations from DOPC:DPPC 2:1 membrane recorded at 220mV and $19°C$ before and after addiction of $2\mu l$ of 15% octanol in methanol solution into the membrane system, which is equivalent to approximately 7.9mol% of anaesthetics in the membrane.

compared with the similar analyses made on neuronal nicotinic acetylcholine receptor (*Zuo et al.*, 2004b) (AChR), which has been used for many years as a model to study the influence of alcohols *Bradley et al.* (1984, 1980); *Forman* (1997); *Forman et al.* (1995); *Forman and Zhou* (1999); *Murrell and Haydon* (1991); *Wood et al.* (1991); *Wu et al.* (1994).
AChR-associated currents, were found to be significantly modulated by octanol, with the single-channel parameters dependent on the alcohol concentration in the membrane - see *fig.*42.

From amplitude histograms in *fig.*42*A* significant change in current amplitude distribution could be seen after octanol was added to the system: the high conductance state levels of amplitude ranging from $100-200pA$ were successively diminished when the molar fraction of octanol in the membrane increased - to around $40pA$ at $7.9mol\%$ to $\sim 15pA$ at $15.9mol\%$ and $\sim 5pA$ at $23.8mol\%$ octanol in the membrane. Dwell time analysis (*fig.*42*B*) also shows that the overall contribution of high conductance channels (level 2, 3) decreases with respect to low conductance states (level 0) when the octanol is present in the membrane, suggesting that the activity of the channels is gradually inhibited. Mean open time of the individual levels did not change significantly, with values ranging from $\sim 3ms$ to $\sim 5ms$.

*Fig.*43 shows the results of amplitude- and dwell time analysis on current fluctuations with different amount of octanol in the membrane, for the two first conductance states of currents (level 1 and level 2). Mean current amplitude $< I_m >$

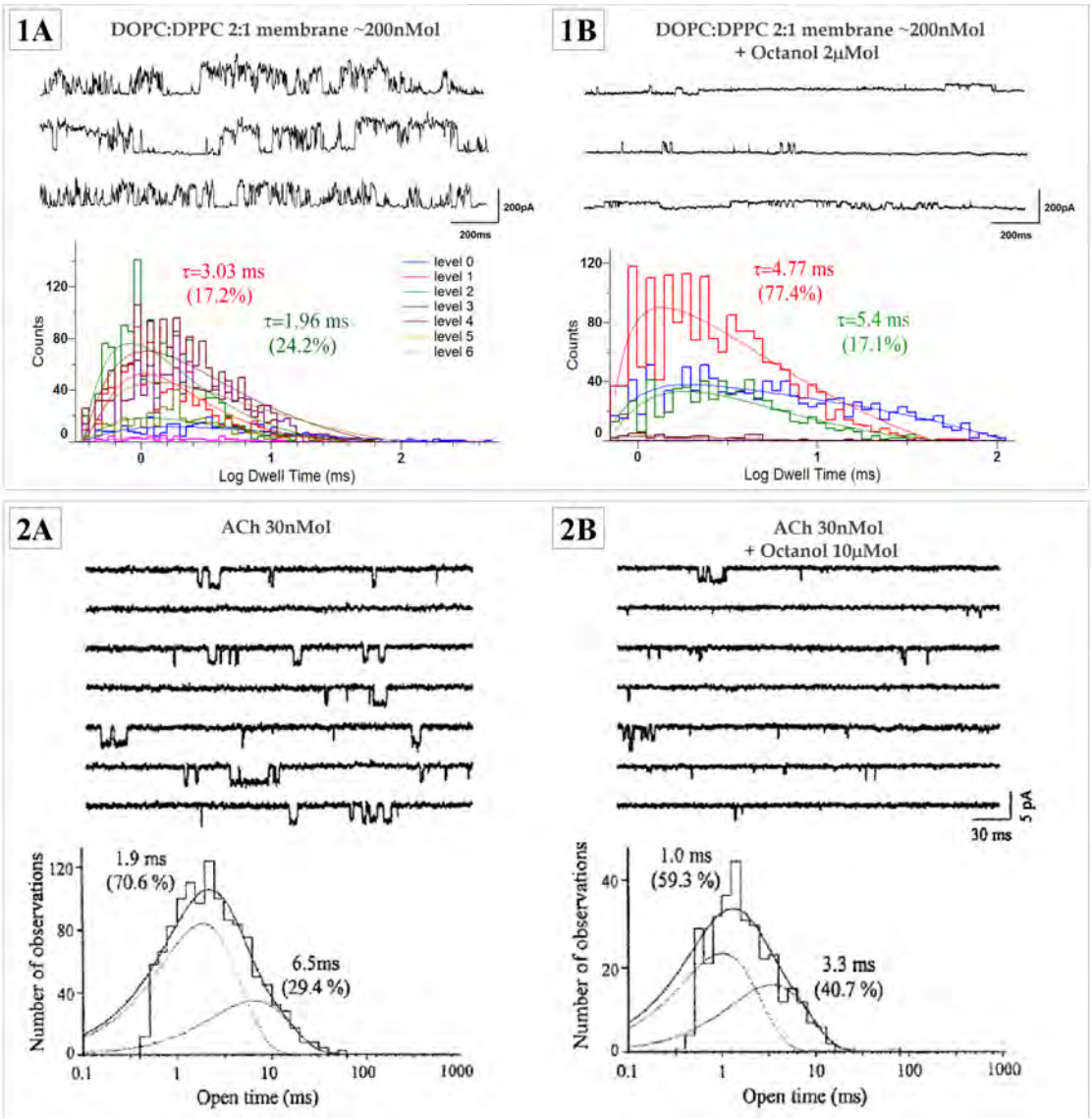


Figure 41: Octanol modulation of pores in DOPC:DPPC membrane (*1A,B*, $\sim 19°C$, $220mV$) compared to the neuronal activity of nicotinic acetylcholine receptor (AChR) induced channels under the influence of octanol (*2A,B*, outide-out patches held at $-70mV$) (*Zuo et al.*, 2004b). In both cases octanol caused a decrease in current fluctuation amplitude. Mean open time for AChR-channels was slightly decreased in the presence of octanol (from$\sim 3.25ms$ to $\sim 1.94ms$) whereas for lipid channels a slightly increasing tendency in dwell-time was observed. Dwell time analysis performed by *Zuo et al.* (2004b) covers only high conductance states, with curves fitted using two time constants, as AChR channel's opening is believed to be characterised by slow and fast time-constant. High conductance state currents in lipid pores are the states above level 2. Percentages (in brackets) show how many channels/pores were open at each conductance state. For the sake of clarity data for only the two lower conductance states are given in *1A* and *1B*. .

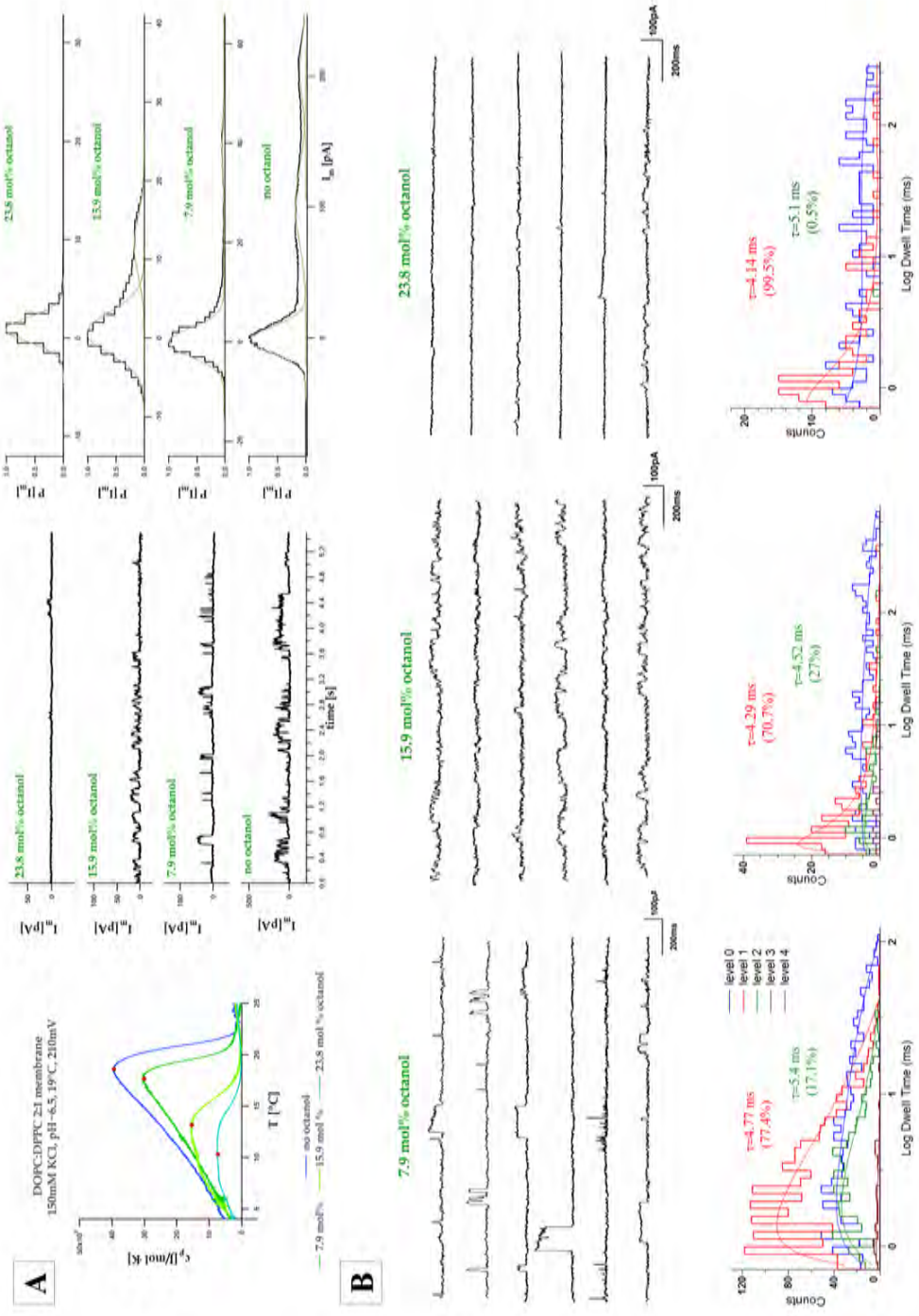


Figure 42: Representative traces with and without a given fraction of octanol in the membrane together with amplitude histograms and mean open time distributions for each case. A shift in heat capacity profile of the lipids constituting the membrane could explain the change in the probability of pore formation when octanol is present.

Molar fraction of octanol in the membrane	P_i [%]	$\langle I_i \rangle$ [pA]	τ_i [ms]
0 mol %	17.15	37.23±15.38	3.03
	24.23	91.74±15.40	1.96
		$\langle I_m \rangle$ =135.48±2.99	$\langle \tau \rangle$ =3.09
7.9 mol %	77.4	6.93±2.82	4.77
	17.1	17.79±5.12	5.4
		$\langle I_m \rangle$ =40.30±2.02	$\langle \tau \rangle$ =5.06
15.9 mol %	70.68	6.73±2.23	4.29
	27.02	13.29±1.43	4.52
		$\langle I_m \rangle$ =6.79±1.20	$\langle \tau \rangle$ =3.72
23.8 mol %	99.46	3.26±0.64	4.14
	0.54	9.125±1.05	5.1
		$\langle I_m \rangle$ =3.79±1.20	$\langle \tau \rangle$ =4.62

Figure 43: Single-ion analysis data gathered from amplitude and dwell-time analysis histograms for different concentrations of octanol in the membrane. Probabilities of pores being open at each conductance state are expressed as a ratio of number of pores present at this state to number of all pores in the membrane. Only data for the the two first states is shown.

was calculated taking all conductance states i into account, as a weighted average of mean current amplitudes $< I_i >$ on each level i:

$$< I_m >= \frac{\sum_i^N P_i < I_i >}{\sum_i^N P_i}$$

where P_i denotes probability of occurrence of a pore at i-level.
From the output of the single channel analysis one could see that the mean membrane current across the membrane devoid octanol decreases significantly in the presence of anaesthetic in the membrane, in a concentration dependent manner. The mean current amplitude of $\sim 135pA$ for the pure lipid membrane drops to around $40pA$ for $7.9mol\%$ octanol in the membrane and the number subsequently gets smaller to $\sim 7pA$ and $\sim 4pA$ for 15.9% and 23.8% molar fraction of octanol, respectively. The decrease in membrane current coincides with the decrease in number of conductance sub-states, which can be seen by increased ratio of probabilities of level 1 with respect to level 2. The mean open times for pores in the membrane $< \tau >$ were not changed markedly with the presence of octanol in the membrane and varied within the range $3 - 5ms$.

Membrane permeability and freezing point depression

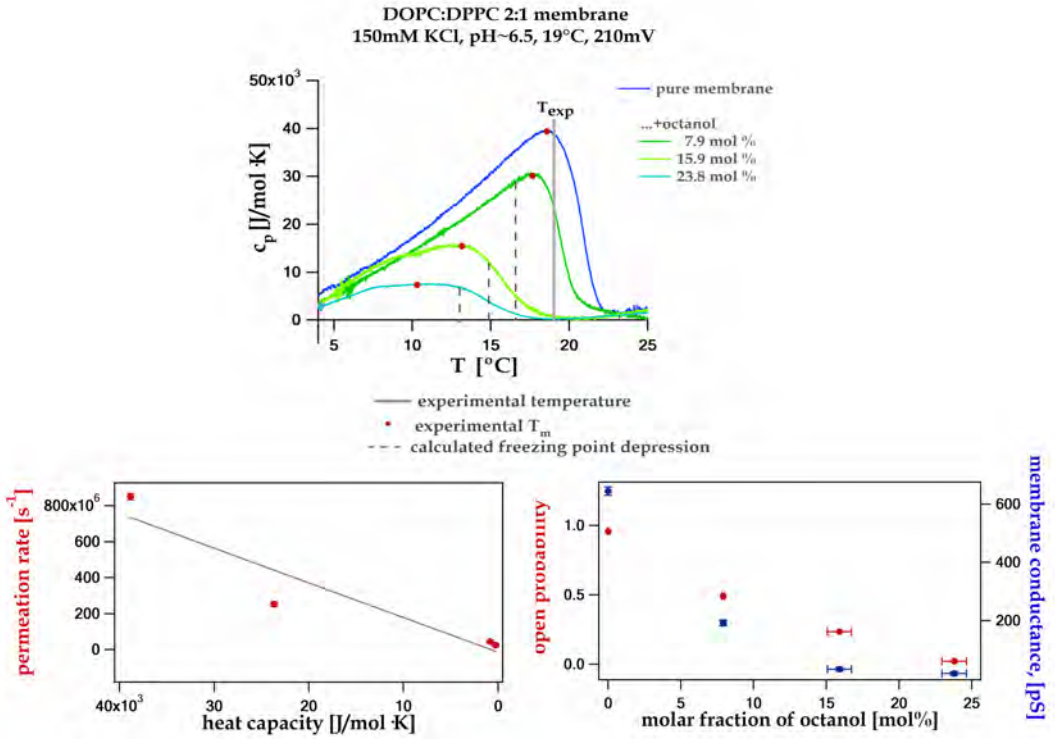
The inhibitory effect of octanol on pore formation in the membrane was shown by decreased open channel probability and decreased mean pore conductance as a function of octanol concentration (*fig.*44).

Decreased open channel probability can be shown by heat capacity profiles of lipids with octanol, which are shifted to the left for higher concentrations of octanol, lowering the likelihood of current fluctuations at the observation temperature, T_{exp}. The theoretical melting temperatures in each case were calculated from the freezing point depression relation and marked as dashed lines in the *fig.*44. The slight differences in values of melting points from theory and the experiments could be due to the preparation of DSC samples and the inaccuracy of calculating the amount of anaesthetics added to the system with respect to molar ratio between lipids and the buffer in the sample.
From the mean membrane currents obtained through single-channel analysis, the membrane permeation rates for each concentration of octanol could be assessed, assuming that a single ion (in our case K^+ or Cl^-) bears single elementary charge $1e = 1.602 \cdot 10^{-19}C$. The permeation rate was decreased substantially when octanol was present in the membrane from around $8.5 \cdot 10^{8 ions/s}$ at 0% fraction of octanol in the membrane, to $\sim 2.5 \cdot 10^{8 ions/s}$, $\sim 4 \cdot 10^{7 ions/s}$ and down to $2.4 \cdot 10^{7 ions/s}$ for $7.9mol\%$, $15.9mol\%$ and $23.8mol\%$ octanol fraction in the membrane, respectively. This was plotted as a function of heat capacity value for each concentration of octanol in the membrane, corresponding to the temperature at which the experiments were carried out ($T_{exp} \backsim 19°C$). The relationship between permeation rate and heat capacity was found to be essentially linear - see*fig.*44.
Table in *fig.*44 summarizes the permeation rates and membrane conductances for each fraction of octanol. Due to the decreased mean transmembrane current $< I_m >$, the mean conductance of open events S_m decreased from around $645 \pm 14pS$ with no octanol in the membrane to $192 \pm 9pS$, $33 \pm 6pS$ and $18 \pm 6pS$ for $7.9mol\%$, $15.9mol\%$ and $23.8mol\%$ fraction of octanol in the membrane respectively.

Ethanol

Ethanol is one of the most widely used psychoactive substances which was found not to be neutral for many ligand-gated ion channels such as N-methyl-D-aspartate (NMDA), serotonin (5-HT(3)), glycine and GABA receptors (*Harris*, 1999; *Harris et al.*, 1995). The inhibitory action of ethanol was also observed in voltage-gated Ca^{2+} channels (*Mullikin-Kilpatrick and Treistman*, 1994) and very recently also in potassium channels, which were seen as very important targets of ethanol (*Brodie et al.*, 2007).

Acetylcholine receptor channels (AChR) were also found to be sensitive to



	Molar fraction of octanol in membrane [mol%]			
	0	7.9	15.9	23.8
$\langle I_m \rangle$	135±2.99	40.30±2.02	6.97±1.20	3.79±1.20
Permeation rate [s^{-1}]	$8.5 \cdot 10^8$	$25.15 \cdot 10^7$	$4.35 \cdot 10^7$	$2.4 \cdot 10^7$
S_m [pS]	645±14	192±9	33±6	18±6

Figure 44: Octanol inhibition to lipid ion channels.
Left: Influence of octanol on melting transition of a DOPC:DPPC 2:1 membrane. Due to the shift of melting profiles to the left caused by the presence of octanol, the open channel probability decreases with the increasing octanol concentration in the membrane (*right top-red*). The decrease in mean membrane current results in a similar relation for membrane conductance (*right top-blue*). Consequently, the permeation rate of ions crossing the membrane containing octanol decreases (*right bottom*). The relation of permeability and the corresponding heat capacity at the temperature of experiment (T_{exp}) is found to be close to linear (compare *fig.13*). .

ethanol, however the contradictory data has been reported showing as if ethanol shortened mean open time in one study (*Nagata et al.*, 1996a) and stabilized channel opening state, i.e. increased mean open duration in others (*Zuo et al.*, 2004a). From the structural similarities in both AChRs and GABA receptors it has been assumed by many that the alcohols would modulate the two types of receptor in the similar manner (*Narahashi et al.*, 1998). However, no such similarities have been found so far, even though the binding pocket for alcohols were already assigned for GABA receptors (*Borghese et al.*, 2002; *Mascia et al.*, 2000).

Among all this controversy around ethanol, the challenge of whether ethanol exhibits any action on lipid pore fluctuations has been undertaken for DOPC:DPPC 2:1 membrane. For the experiments with ethanol, 99% alcohol was added symmetrically on both sides of the membrane under 120mV transmembrane potential and a temperature of $\sim 19°C$.
Similarly to the experiments with octanol, the results presented below come from BLM experiments performed on one lipid bilayer, the thickness of which was estimated (from the capacitance measurement) to be of the order of ∼3.1 nm.

Amplitude and open time distribution analyses

The amplitude and dwell-time distribution analyses have shown that ethanol does influence the activity of lipid ion channels. Whereas the mean amplitude of current fluctuations does not seem to be significantly affected, we do observe a decrease in the number of conductance states, and moreover, the mean open time is increased with increasing fraction of ethanol in the membrane (*fig.*45). With the mean amplitude of current fluctuations around $\sim 50pA$, the mean open time for pores increases from $\sim 4ms$ for $0mol\%$ ethanol in the membrane to $\sim 12ms$ for $9.5mol\%$ and $\sim 18ms$ for $19.5mol\%$ ethanol in the membrane. Similar findings were reported for experiments with ACh-receptor channels subjected to 100mM of ethanol (*Zuo et al.*, 2004a). Tte amount of ethanol used in BLM experiments was 1mM and 2mM for $9.5mol\%$ and $19.5mol\%$, respectively.

*Fig.*46 presents statistical data obtained from single-channel analysis of lipid current fluctuations in BLMs with ethanol.

Membrane permeability and freezing point depression

The inhibitory effect of ethanol on pore formation could also be seen as a decreased open channel probability and decreased pore conductance as a function of octanol concentration (*fig.*47).

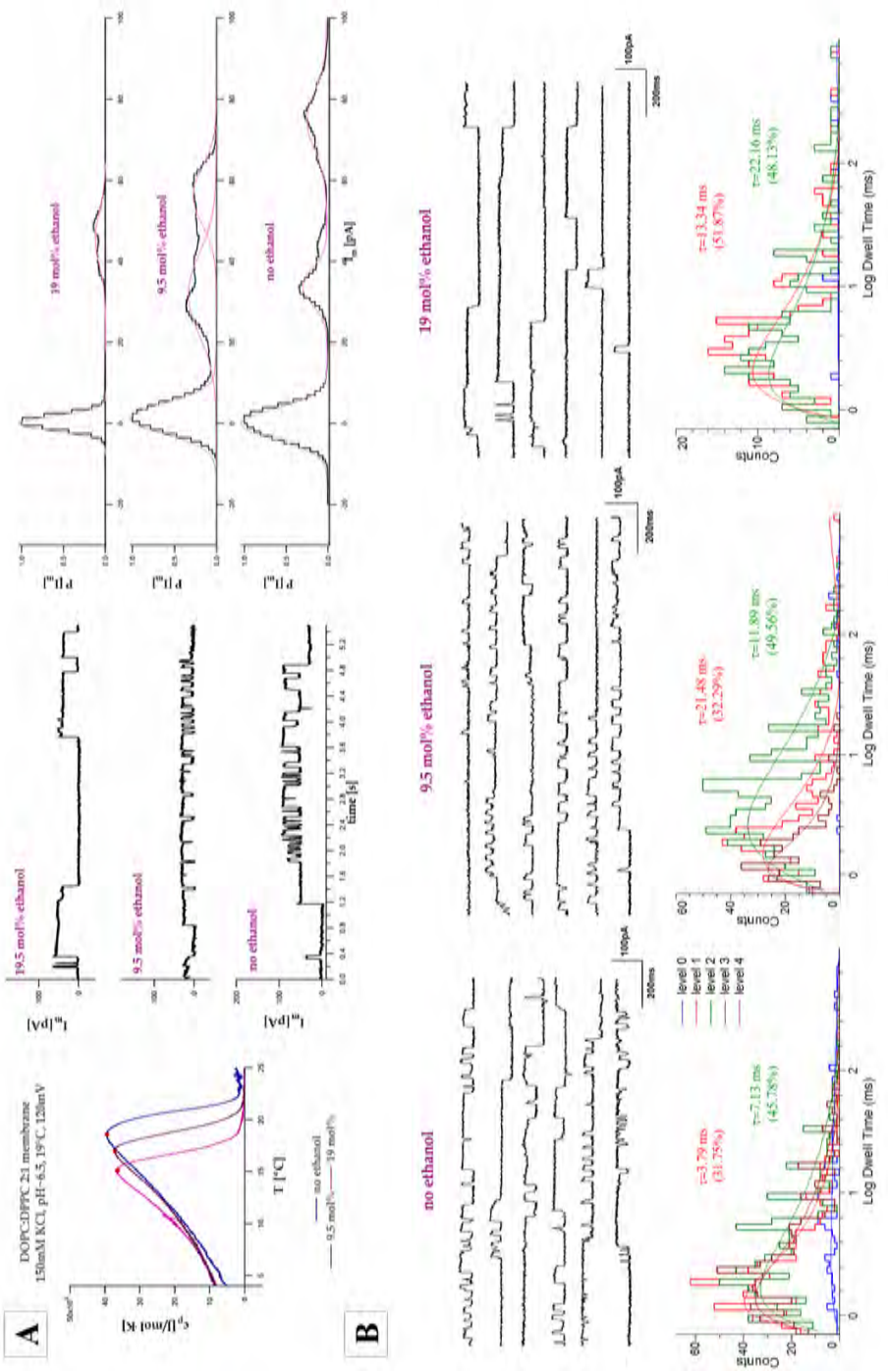


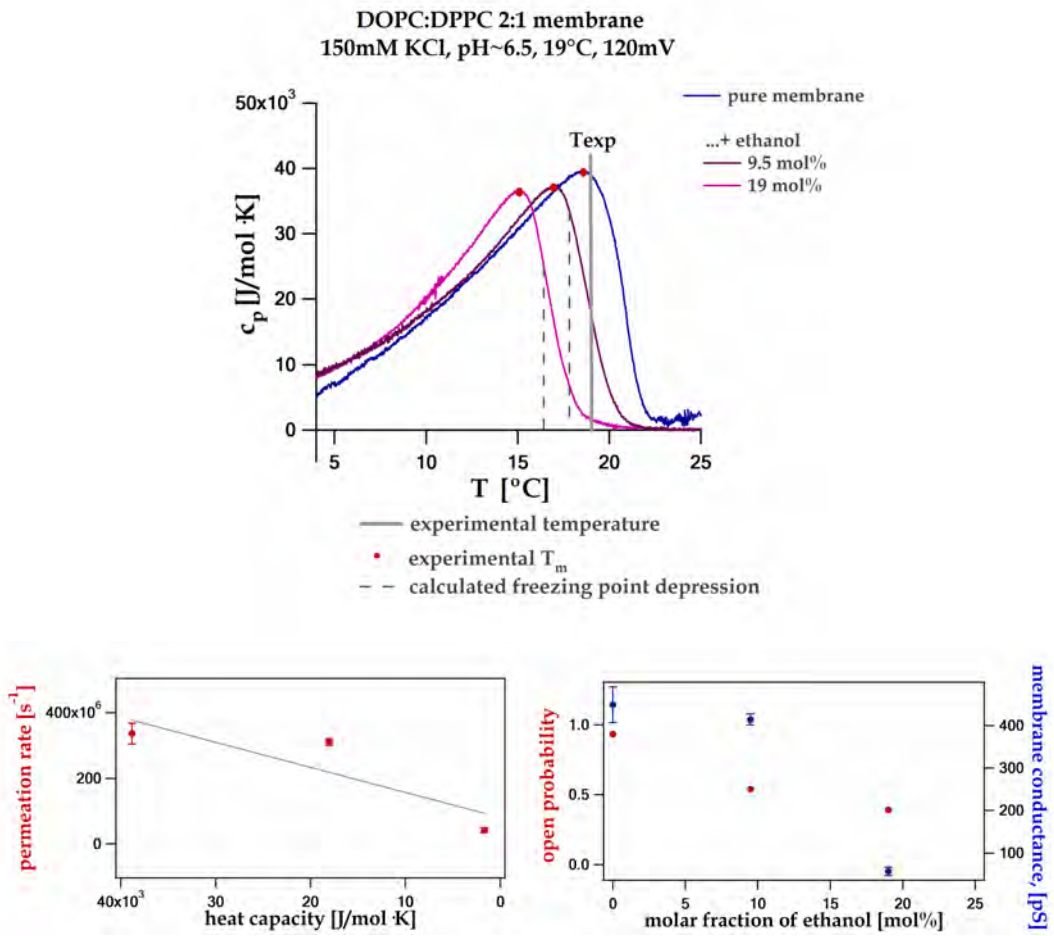
Figure 45: Representative traces without and with given fraction of ethanol in the membrane together with amplitude histograms and mean open time distributions for each case. A slight shift in heat capacity profile of lipids constituting the membrane could explain the perturbed probability in pore formation with the presence of ethanol content in the membrane.

Molar fraction of ethanol in the membrane	P_i [%]	$\langle I_i \rangle$ [pA]	τ_i [ms]
0 mol %	31.75	37.64±6.01	3.79
	45.78	52.86±10.09	7.13
	19.38	73.89±3.29	5.69
		$\langle I_m \rangle$ =53.89±5.04	$\langle \tau \rangle$ =4.41
9.5 mol %	32.29	39.12±3.44	21.48
	49.56	51.29±7.27	11.89
	18.15	64.24±1.13	2.05
		$\langle I_m \rangle$ =49.71±3.78	$\langle \tau \rangle$ =11.81
19.5 mol %	51.87	40.60±3.32	13.34
	48.13	45.57±2.51	22.16
		$\langle I_m \rangle$ =42.99±2.10	$\langle \tau \rangle$ =17.75

Figure 46: Single-ion analysis data gathered from amplitude and dwell-time analysis histograms for different concentrations of ethanol in the membrane. Probabilities of pores open at each conductance state are expressed as a ratio of number of pores present at this state to number of all pores in the membrane. Only data for the the three first states is shown (only two sub-conductance levels were present with 19mol% ethanol in the membrane).

Decreased open channel probability can be explained from lipid heat capacity profiles with ethanol, which are shifted to the left for higher concentrations of ethanol, consequently lowering the likelihood of current fluctuations at the temperature of observations T_m. The theoretical melting temperatures in each case were calculated from the freezing point depression relation and marked as dashed lines in the *fig.47*. The heat capacity profiles are only shifted $1 - 2°C$ from one another, due to over 400-fold lower partition coefficient of ethanol as compared to that of octanol (*Jain and Wray*, 1978; *Firestone et al.*, 1986).

Table in *fig.47*, summarizes mean pore conductance change with different amounts of ethanol in the membrane and the average permeation rates, calculated from the mean transmembrane current $< I_m >$. The average conductance of the membrane pores S_m decreased only slightly when ethanol was present in the system: from $441 \pm 42pS$ for ethanol-free lipid membrane to $414 \pm 31pS$ for $9.5mol\%$ of ethanol and $359 \pm 18pS$ for $19.5mol\%$of ethanol in the membrane. The permeation rate was reduced from $3.36 \cdot 10^{8 ions}/_s$ for $0mol\%$ fraction of ethanol to $3.1 \cdot 10^{8 ions}/_s$ and $4.2 \cdot 10^{7 ions}/_s$ for $9.5mol\%$ and $19.5mol\%$ concentration of ethanol in themembrane. Again, similarly to the findings with octanol, the permeation rate was found to be in approximate linear relation to heat capacity (compare *fig.13*).



	Molar fraction of ethanol in membrane [mol%]		
	0	9.5	19.5
$<I_m>$	53.89±5.04	49.72±3.78	42.99±2.10
Permeation rate [s^{-1}]	$3.36 \cdot 10^8$	$3.1 \cdot 10^8$	$4.2 \cdot 10^7$
S_m [pS]	441±42	414±31	359±18

Figure 47: Ethanol inhibition to lipid ion channels.
Left: Influence of ethanol on melting transition od DOPC:DPPC 2:1 membrane. Due to the shift of melting profiles to the left in the presence of ethanol, the open channel probability decreases with the increasing ethanol concentration in the membrane (*right top-red*). The decrease in mean membrane current results in a similar relation for membrane conductance (*right top-blue*). Consequently, permeation rate of ions crossing the membrane containing octanol decreases (*right bottom*). The relationship between permeability and the corresponding heat capacity at the temperature of experiment (T_{exp}) is found to be close to linear (compare *fig.13*)

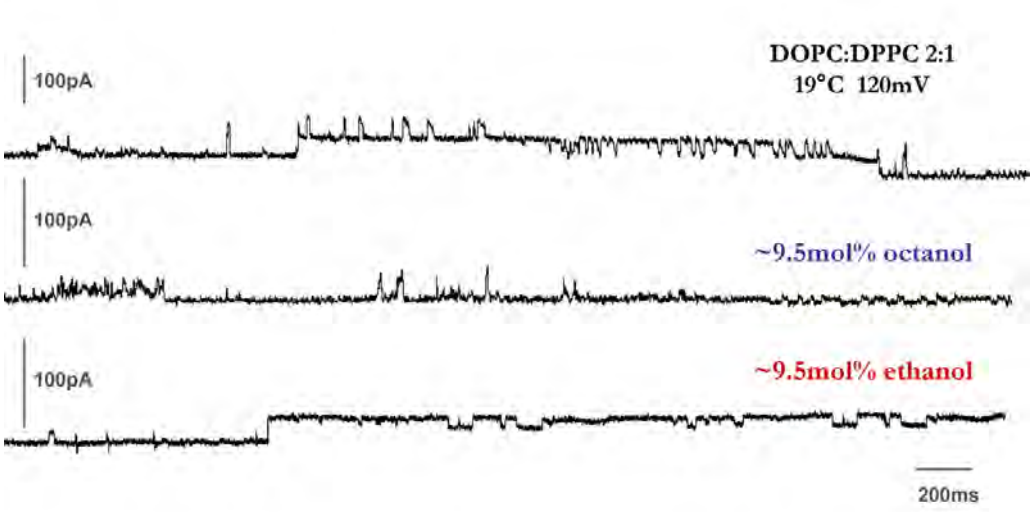


Figure 48: Representative current traces of DOPC:DPPC 2:1 membrane without any anaethetics in the membrane (*top*) and with similar content of octanol (*middle*) and ethanol (*bottom*). The experiments were made under the same conditions of transmembrane voltage ($120mV$) and temperature ($\sim 19°C$). From capacitance tests membrane thickness was calculated to be in the order of 3.6nm.

Mechanism of anaesthesia on lipid pores

*Fig.*49 summarizes the effect of octanol and ethanol on current fluctuations in pure lipid (DOPC:DPPC) membrane, composed of DOPC:DPPC 2:1 lipid mixture. The inhibitory effect of both anaesthetics can be successfully explained by the shift in melting profiles of lipids that they introduce, thus lowering the probability of observing current fluctuations. The specific effect that both of the alcohols introduce into lipid current fluctuations are slightly different. Octanol was seen to decrease the mean membrane current amplitude and have a weak influence on mean open time of pores, whereas ethanol increased the mean dwell time of channels, with only a slight change in membrane current amplitude. Both anaesthetics however were observed to have inhibitory effect on lipid pore formation in the membrane (*fig.*49)

*Fig.*48 shows representative current traces from DOPC:DPPC 2:1 membrane, which was subjected to the influence of the two anaesthetics, so that in the end the molar fraction of both of them was approximately the same and equal to $\sim 9.5mol\%$. From the different modulation of current traces by the two alcohols, the two different modes of action of octanol and ethanol on lipid membrane pores can be deducted.

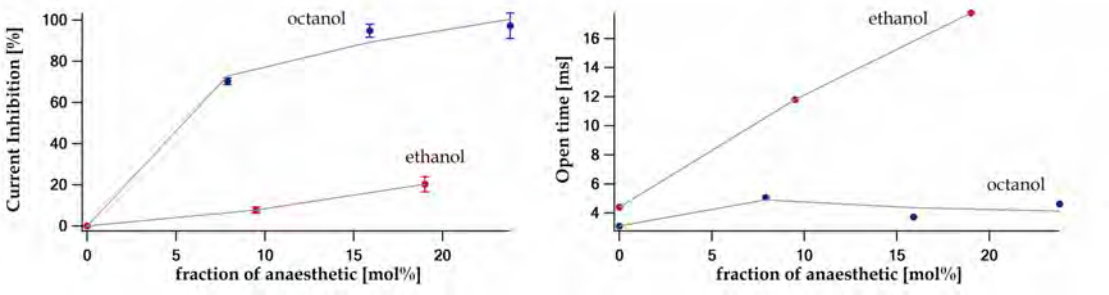


Figure 49: Both octanol and ethanol changed the mean open probability of lipid channels in the membrane. Due to the higher partition coefficient of octanol compared to that of ethanol, octanol changed membrane conductance and thus membrane permeability more significantly than ethanol did. Ethanol, on the other hand, was observed to affect the mean open time of the channels more than octanol, which barely influenced mean opening time distributions. In literature, the effect of anaesthetics on ion channels is interpreted both in terms of current and mean dwell time change.

4.2.3 pH

Following observations made by *Tyauble et al.* (1976), that the lipid phase transition temperature can be shifted by a change in pH of external solution, experiments were designed to check this dependence on BLM membranes in terms of spontaneous current fluctuations.

The BLM experiments were first performed on uncharged, DOPC:DPPC 2:1 lipid mixture, the same which was successfully used for previous experiments with anaethetics. The measurements were performed in a way that the membrane solution was titrated by 0.1M HCl/0.1M NaCl during the BLM recording, i.e. on the same membrane, in quantities predicted beforehand from titration experiments on the lipid solution in the buffer. The pH was also double-checked after the BLM recordings had been finished in the teflon chamber directly. At each pH value the current trace was recorded for approximately 10 runs (i.e. 300s) before the pH was changed to a different value.

DOPC:DPPC 2:1 membranes

A shift in pH of membrane solution did cause an observable change in current fluctuation pattern, diminishing the fluctuations at lower pHs and prolonging mean opening times at higher pHs (*fig.*50*top*). However, the heat capacity profiles of lipids at different pH of the solution were not significantly changed, with respect to main melting temperature of the lipids in unbuffered KCl of $pH \sim 6.5$ (*fig.*50*bottom*).

DLPC:DMPC 2:1 membranes

Similar experiments were carried out on the DLPC:DMPC 2:1 mixture, which in water has a broad melting transition, in a range of $3-11°C$. After DLPC:DMPC membrane had been created, the pH of the 150mM KCl was lowered gradually in an attempt to see some current fluctuations, which, if visible, would point to the fact that membrane entered its melting transition regime. Preliminary experiments showed that single current steps appear at very acidic pH of the solution. This was further confirmed by DSC profile of DLPC:DMPC 2:1 mixture in KCl-HCl buffer of pH=2 (*fig.*51), which was moved from the low temperature range towards higher temperatures the room temperature of $19°C$ would fall into.

DPPC membranes

Experiments on DPPC membranes kept at very basic pHs ($pH \sim 11$) did not seem to induce any current fluctuations, which can be interpreted such that the pH change of $\triangle pH = 4$, from the neutral value to the value used in the experiments, could not shift the melting transition of DPPC of $\sim 41°C$ (compare *fig.58, sec.Appendix)* to the temperature at which the experiments were performed ($\sim 20°C$) and by that increase the probability of pore formation in the membrane. Indeed, the DSC profile of DPPC at $pH \sim 11$ (not shown) did not change significantly from the heat capacity profile of the aqueous suspension of the lipid.

Some experiments. however, on how protons may leak through the DPPC membrane were done by adding 0.1M HCl on one side of the membrane only. By disturbing the acidity of the KCl solution on one side of the membrane some step-like current fluctuations were seen with a membrane potential 110mV at a temperature of $21°C$. The current amplitudes were even more pronounced when the amount of of hydrochloric acid was increased from $30\mu mol$ to $90\mu mol$ - the ampitude analysis shown that the mean current amplitude increased from 15.3pA to 25.1pA for $30\mu mol$ and $90\mu mol$ of HCl acid, respectively. Dwell-time histograms in *fig.*51 moreover show that the ratio of higher sub-conductance states with respect to lower conductance states increased with the increase of proton concentration on one side of the membrane and that the mean open time for channels shortened for higher molar concentration of protons.

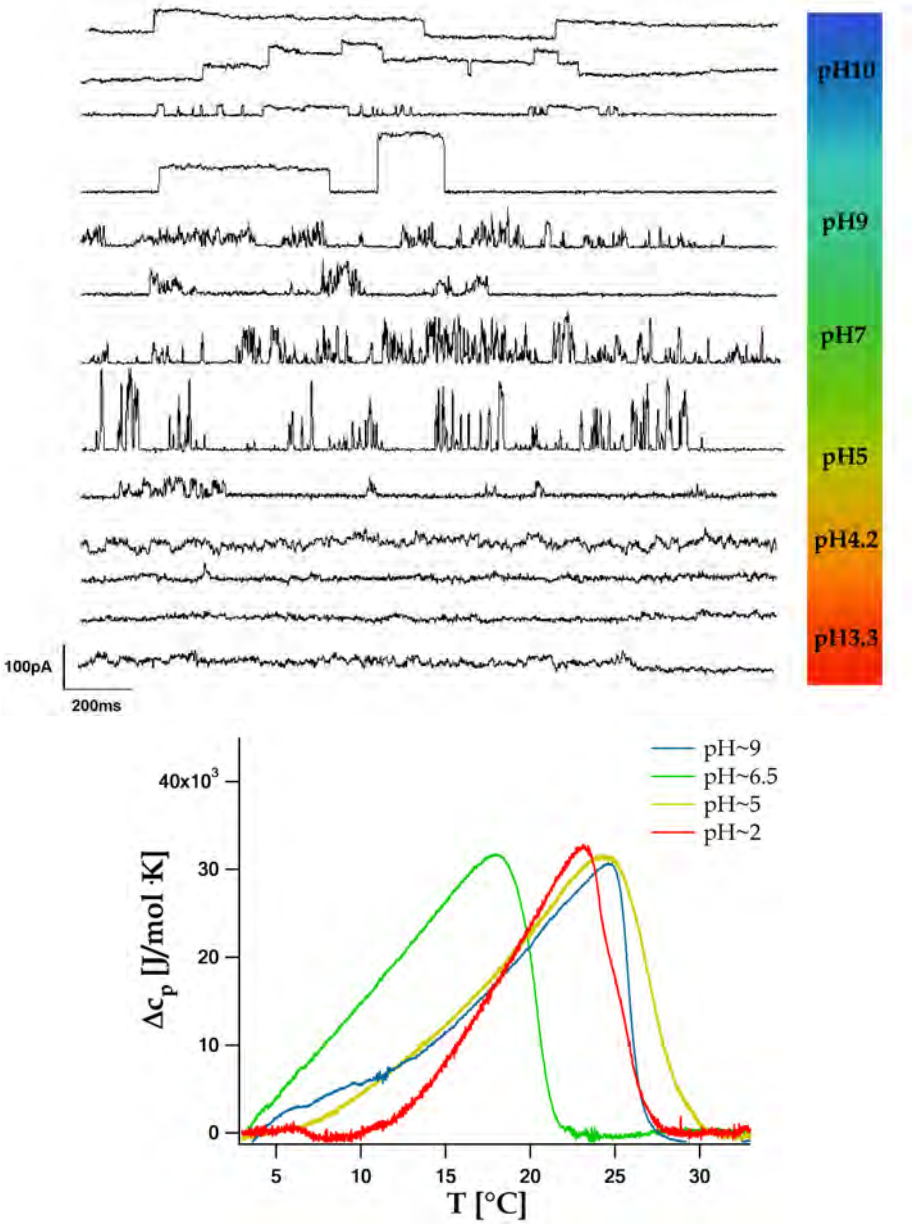


Figure 50: *Top*: cuurent fluctuations of DOPC:DPPC 2:1 membrane at different pHs of external solution (membrane clamped at 120mV, 20.2°C). General tendency for decreasing current amplitude and shortening channel open times has been observed for lower pH values and appearance of longer openings was observed at higher pHs. Inconsistency in heat capacity profiles (*bottom*) does not seem to explain the pore behaviour at different pHs successfully. For the DSC scans the buffers HEPES, acetate and KCl-HCl were used for pH7, 5 and 2, respectively. Membrane solution 150mM KCl was titrated with 0.1M NaCl to arrive at pH∼11.

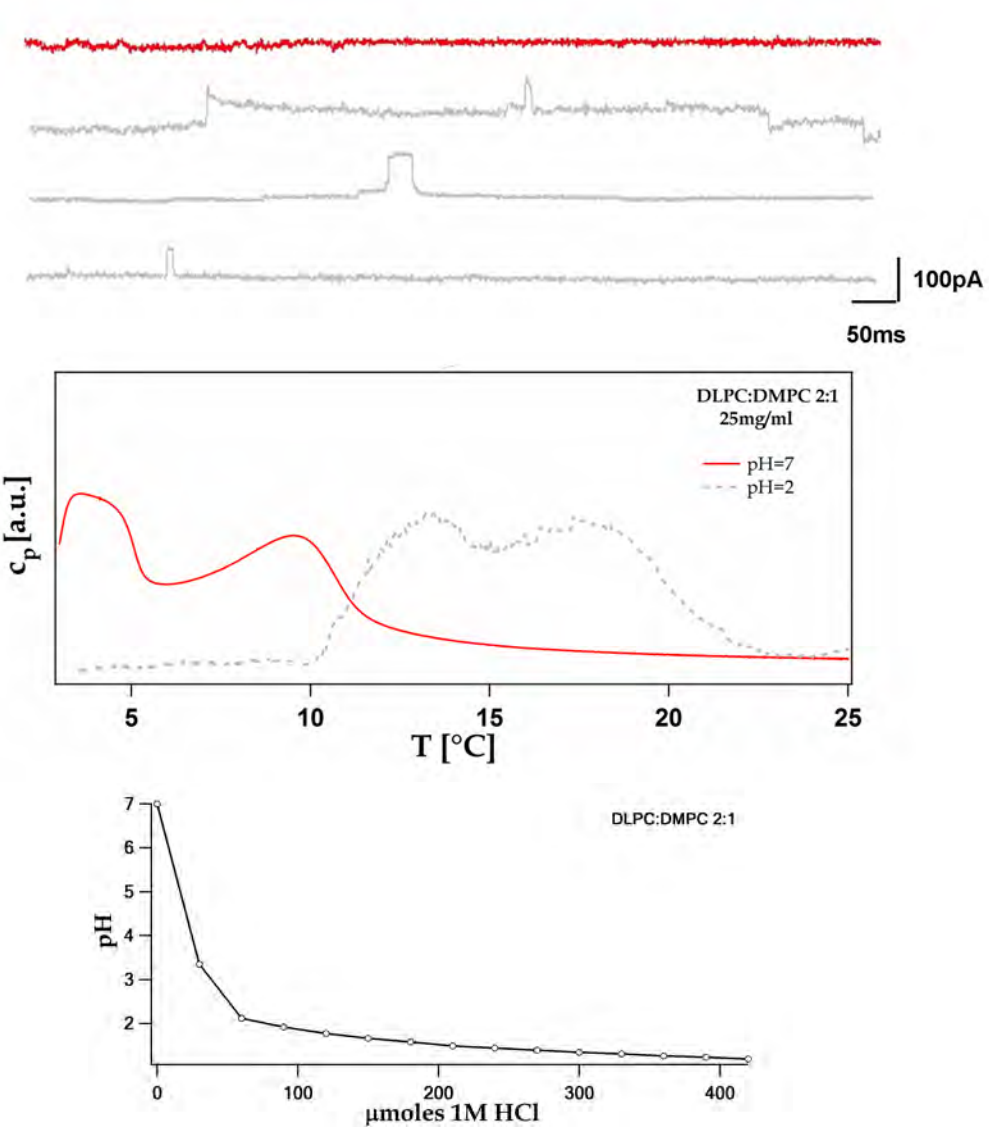


Figure 51: *Middle*: Heat capacity profile of DLPC:DMPC 2:1mixture (scaled) in pH=7 (red) and pH=2 (grey). Corresponding current traces were recorded at 19°C with membrane potential 90mV (*top*). 150mM KCl solution was titrated to pH=2 in the course of experiments with 0.1M HCl and the amounts of hydrochloric acid required to go down to the desired pH value were estimated through simulation titration experiments before the BLM measurements were conducted (*bottom*). The pH of the membrane solution was re-checked after the BLM recordings in the teflon chamber were finished. Initial membrane thickness was calculated to be in the order of 4.3nm.

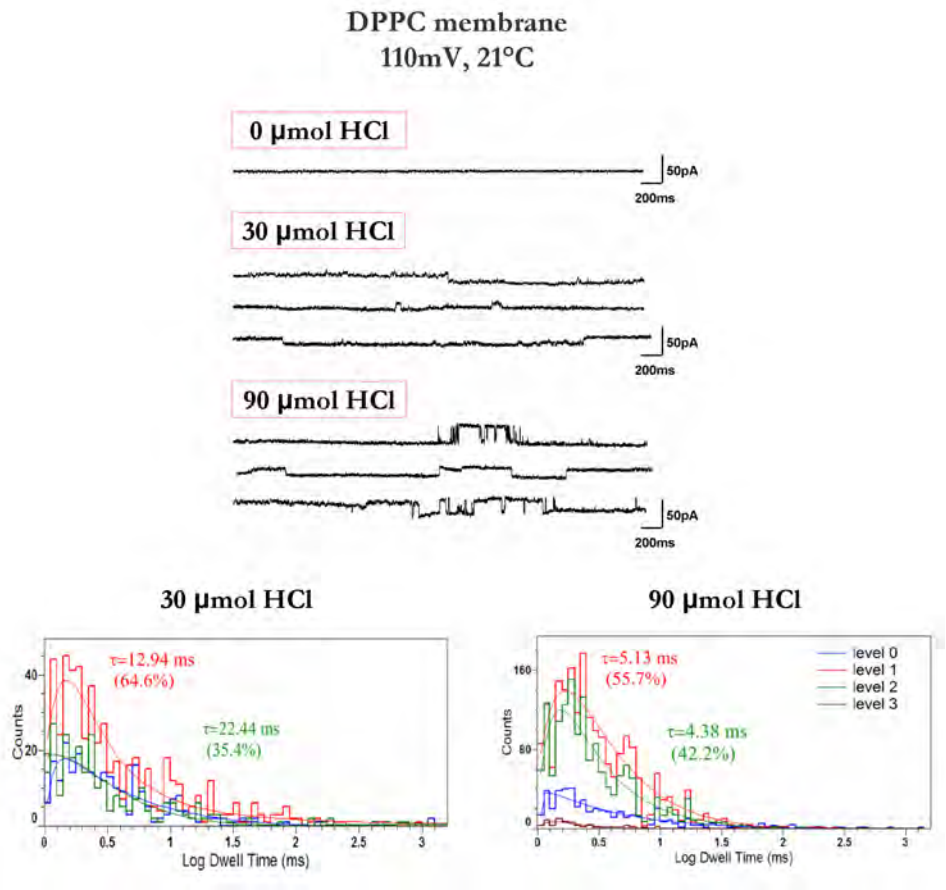


Figure 52: Current fluctuations from DPPC membrane were observed when the concentration of protons was increased on one side of the membrane, next to the positive electrode. The dwell time-analysis indicated that the higher conductance state (level 2) was even more pronounced with 90μmol (42%) than with 30μmol of HCl added (35.4%), which at the same time decreased the overall percentage of lower conductance sub-states (level1). Mean open time of pores also shortened significantly at higher proton concentration - from around 13ms and 22ms for levels 1 and 2 for 30μmol of HCl to 5ms and 4ms for 90μmol HCl. From the DSC scan of DPPC taken at pH=11 no change of the main melting temperature was seen, only the shape of the profile was slightly disturbed (thermogram not shown here).

5 Future perspectives

It is believed that in the future BLM experiments on more complex lipid systems could be performed. Studying properties of membranes made of charged lipids and/or applying different buffer solutions on both sides of the membrane could further allow studies on BLMs' ion selectivity. The ion selectivities could then be used to determine the relative permeabilities of pores to different ion types. Moreover, the experiments with pore-forming proteins, such as gramicidin A could be designed in order to define the contribution of the membrane permeability from the lipid matrix membrane alone in comparison to the permeability induced by the protein species.

It is also hoped that the experiments on pure lipid membranes could be performed by patch-clamp technique. Preliminary attempts to create a membrane under the patch clamp conditions resulted in gigaOhm seal resistances lasting only a few seconds, after which the supposedly formed membrane disrupted.

In order to address the question of mechanism of pore formation in lipid membranes, fluorescence microscopy experiments could be relevant, allowing direct observation of lipid domain rearrangements in the melting transition. Since macroscopic domain separation is believed to be the largest at the maximum of heat capacity profiles, i.e. in the main melting transition of lipids, observation of behaviour of the domains could let us explain the maximum probability of pore formation in that temperature regime. However, a problem that might occur is that the microscopic observational volume of a μm-scale would most likely not allow to detect all the lipid domains individually, as they are often in nm-range. The experimental set up would furthermore need to be arranged such that the current fluctuations across the membrane could be recorded while the microscopy images are taken.

Fluorescence Correlation Spectroscopy could also be applied to pure lipid bilayer membranes and a degree of fluorescence intensity fluctuations, corresponding to the lipid state fluctuations in the lipid phase transitions could be assesed. This approach was already undertaken by Konrad Kaufmann in the 1980's and brought promising preliminary results. It would however require fluorescent labels which would have to be sensitive to the lipid environment (gel/fluid state) and, again, the size of the fluctuations would need to be on at least tenths of μm scale to allow a reasonable good detection of the signal fluorescence intensity.

The question of how an anaesthetic exert their actions on the lipid pore activity on a molecular level could also be of interest. To investigate this interaction the fluorescence microscope technique could also be found useful.

Furthermore, experiments with a wider range of anaesthetics would need to

be planned, possibly with some more clinically-oriented drugs, in order to obtain a better picture of how anaesthesia work on lipid membranes. Aspirin, for instance, could be a good candidate to start with, as it has shown to decrease currents in transmembrane protein acting as chloride channel (*Tondelier et al.*, 1999) and has already been proved to shift the melting profile of lipids substantially (*Ebel*, 1999). It is therefore suspected that the drug would also have a significant effect on lipid pore activity. Another example would be lidocaine, which has been proved to affect activity of not only sodium ion channel in the heart (*Bean et al.*, 1983), but also potassium and calcium voltage-dependent channels (*Josephson*, 1988). Other drugs could also be of interest, especially when their effect with different pHs is concerned, such as paracetamol and ibuprofen or lignocaine, to name a few. Their solubility profiles were found to depend on pH of the environment (*Shaw et al.*, 2005). *Hadgraft and Valenta* (2000) also reported that permeation of ionized drugs through lipophilic pathways is strongly pH-dependent and the maximum flux may occur at a pH where ionization is high, as solubility of ionized material is significantly higher than unionized.

Of additional interest would be an approach to determine how the lipid membranes respond to the mixture of some anaesthetics added to the system and check whether the potency of the individual drugs is additive (which was already suggested by Ernst Overton over a century ago).

6 Summary and discussion

This work explores the concept of ion-channel like current fluctuations in pure lipid membranes, which was initiated almost three decades ago (*Yafuso et al.*, 1974; *Antonov et al.*, 1980; *Kaufmann et al.*, 1989, etc.).

It was shown that permeability of unmodified lipid membrane, strongly depends on the thermodynamic state of the system, which is in turn governed by temperature, voltage, pH of the ambient buffer and the presence of anaesthetics in the membrane. The membrane permeability is measured by the degree of electric current fluctuations when the transmembrane potential is applied with BLM technique.

These step-wise changes in current observed were attributed to pores in the membrane, in accordance with the hydrophilic-pore hypotheses as a mechanism for membrane transport (*Doniach*, 1978; *Kaufmann et al.*, 1989; *Nagle and Scott*, 1978) and they were analysed with a single-channel analysis approach, which had been developed previously for the analysis of protein ion channel data (*Neher and Sakmann*, 1976). In thermodynamic terms, a lipid pore represents nothing more than an allowable state of the membrane, characterised by a finite probability of occurence. The current fluctuations, which are due to the pore formation in the membrane, are at their greatest in the lipid phase transition regime. The probability of pore formation is therefore believed to be at a maximum during the lipid melting process.

Lipid pores are facts

Discrete current fluctuations were observed for membranes composed of both single (DMPC, DPPC, DPhPC) and binary lipid mixtures (DOPC:DPPC, DLPM:DMPC) in their melting temperature regimes. No currents were observed away from the melting transition conditions. The current fluctuations were found to have a quantised, step-like character. Fluctuations occured in the form of both short, isolated openings (spikes and steps) and in a series of bursts, where an enhanced activity (flickering) of pores was seen during some time intervals, while no activity was seen in other periods.
Through single-ion channel analysis current amplitude and mean lifetime distributions were obtained. Current fluctuations were measured at a picoAmpere level and on a millisecond time scale. These are of the same order of magnitude as physiological measurements on protein ion channels. Amplitude histograms moreover highlighted the descrete and multi-level conductance nature of these fluctuations. While no explanation has been found so far to justify quantitization in current steps for either proteins or for lipid channels, the multilevel conductance levels for proteins are assumed to arise from changes in confor-

mational states of molecules when conducting ions (e.g. (*Bamberg and auger*, 1973)). However, if one applies a similar interpretation of conductance states to proteins as to lipid pores, it can also be assumed that multiconductance states might indicate more than one channel in the membrane patch present.
From the mean amplitudes of transmembrane currents membrane permeability rates were estimated to be in the order of $10^7 - 10^8 ions/s$, which is of the same order of magnitude as the rates of ion passage in protein ion channels (*Hille*, 2001). Moreover, from the experiments with anaesthetics, the permeability rates for pores in DOPC:DPPC membrane were found to stay in a proportional relation to the heat capacity values (see *fig.44*, *47*). Similar results was obtained by *Blicher* (2007) on the example of DPPC vesicles (see *fig.13*).

The opening times for pores in both protein-free and biological membrane types are in the range of a few milliseconds. It is tempting to assume that this is related to the process of domain formation in biomembranes, which is characterised by relaxation times of a similar order of magnitude. In lipid melting transitions the relaxation times of lipid systems were found to be proportional to the excess heat capacity (*Seeger et al.*, 2007; *Seeger*, 2006; *Grabitz et al.*, 2002), which made it possible to estimate them from the lipid calorimetric profiles. From the experiments on DPPC and DMPC membranes, clamped under the same voltage conditions, it has been noted that pores in DPPC membranes were characterized by mean opening times approximately four-times longer than the DMPC pores. This could be in agreement with the maximum of the heat capacity values for both lipids (see *fig.33*). The BLM experiments with DPPC and DMPC bilayers were not performed in sufficient number, due to the difficulties in adjusting the temperature to the exact melting points of lipids. The heat capacity profiles of both DPPC and DMPC are very narrow, as the melting processes of the lipids are strongly cooperative. Therefore, more experiments need to be planned to define the relationship between channel opening and relaxation times in future.

Pore formation in the pure lipid membranes was shown to be characterised by the linear current-voltage dependence in the phase transition regime over a wide range of voltages (*fig.34*). An increase in membrane conductivity seems to be present above a threshold voltage of 50-60 mV, which might correspond to the minimum pore radius detectable. The threshold voltage can however also depend on the temperature at which the experiments were performed, and how far it is from the main transition temperature of lipids under consideration. A similar threshold phenomenon was previously suggested in 1974 by *Yafuso et al.* (1974) when the discrete current fluctuations were first observed in oxidized cholesterol black lipid membranes. Non-linear behaviour at voltages

around 200mV could suggest that at this point the pore formation process is driven not only by the melting transition but also by the applied transmembrane voltage and 'lipid channels' become not only temperature-gated but also voltage-sensitive.

From the I-V relation the average pore conductance in DOPC:DPPC membranes was calculated to be $\sim 300 \pm 80pS$. The mean conductances of potassium ion channels reported in the literature vary from few pS to 240pS (*Hille*, 2001).

Usually, due to the non-zero concentration gradient across a biological membrane the reversal potential is not equal to the equilibrium (Nernst) potential for a protein channel (see also *section Appendix*). This however, can be assumed to be the case with the lipid pores data reported here. Nevertheless, due to zero reversal potential, the I-V plot is inconclusive in the identification of the ion species which the lipid pores are permeant to. Some considerations about the permeant ions can be made based on the lipid pore size estimation as compared to the hydrodynamic sizes of $K^+$and Cl^- ions (see further). In order to determine the lipid channel selectivity to ions, membranes with asymmetric solutions on either side of the membrane would need to be used or membranes made of charged lipids would need to be investigated. Such experiments, already conducted by Antonov (*Antonov et al.*, 1980) suggested that positively charged membranes would induce anionic selectivity. It would be expected that the reversal potential is a function of the relative permeabilities for each ion and the equilibrium potentials of the ions.

The non-linear part in the I-V plot can indicate electroporation of the lipid membrane. The process of creating pores in response to high electric fields, referred to as membrane electroporation has been a subject of research since the early 1980's (*Neumann and Rosenheck*, 1972) and it is nowadays of interest to both physicists and clinicists as a very powerful tool for gene transfer or drug delivery into cells and tissues (*Mir et al.*, 1995; *Dev and Hofmann*, 1994; *Mir*, 2008). For this reason however, significantly higher voltages are used ($0.6 - 1.1V$), favouring strong current fluctuations in the lipid membranes. Following Antonov et al. (*Antonov et al.*, 2005), one could therefore refer to lipid pores observed in melting transition temperatures at reasonably low voltages as the result of 'soft perforation' of lipid membranes, in contrast to electroporation which usually happens at significantly higher transmembrane voltages.

Considerations about the influence of voltage on membranes have made us suspect that the transmembrane voltage can move the lipids into the phase transition and thus induce channel-like fluctuations even if the membrane is not initially in the melting temperature range. Some preliminary experiments in this area have been performed by *Antonov et al.* (1990), who reported that a

voltage increase of 150mV can lead to a rise in the phase transition temperature of phosphatic acids (DPPA) by $8 - 12°C$, but cause only slight increase in the melting temperature of DPPC or DSPC lipids. Others (*Cotterill*, 1978) report that field changes of $10^7 V/m$ can induce a change of $2K$ in the transition temperature of DPPC membranes. One could suppose that the the electric field can introduce a change of lipid phase by rearranging the lipid headgroups and thus the electric interations between them (see*fig.*53). This situation can well be imagined to happen for lipid monolayers, but more complicated interactions could take place in bilayer membranes. The problem is undoubtly worth exploring in future research. Some qualitative considerations about voltage as a factor introducing the lipids into their melting transitions are presented in *section Appendix*, on the example of DPhPC and DPPC membranes.

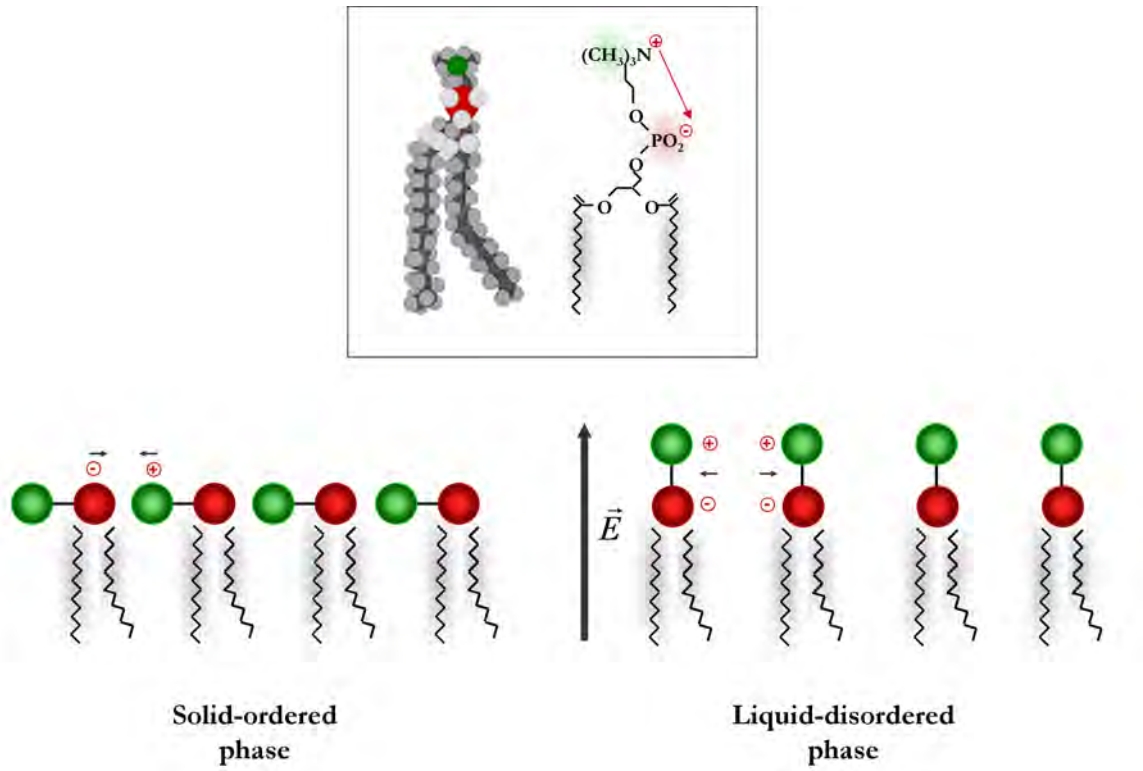


Figure 53: A single phospholipid head group consists of positively charged choline bound to negatively charged phosphate group, which makes lipid molecules own characteristics of electric dipoles (*top*). In lipid monolayers, the head groups will be aligned with the direction of an external electric field. The electric interations between the dipoles might induce a change in lipid phase from solid-ordered to liquid disordered (*bottom*). This might suggest a possible mechanism of how the electric field might introduce the lipid melting transition in monolayers. In a lipid bilayer, however, the opposite effects of electric field on the lipid state might be expected in one layer with respect to the other.

The main lipid pore size was derived from a simple model, in which lipid pores can be described as electrolyte-filled cylindrical tubes (*Antonov et al.*, 2005). The average pore radius for DOPC:DPPC 2:1 membrane was estimated as $0.75 \pm 0.11nm$. This value is in the same order of magnitude as the average size of lipid molecule. For DPPC lipid, for instance, from the mean area per lipid molecule in the gel state $0.474nm^2$ and the mean area per lipid molecule in the fluid state $0.629nm^2$ (*Heimburg*, 1998), the averge size of the single lipid could be estimated as $\sim 0.69nm$ for the gel phase and $\sim 0.79nm$ for the lipid phase. Furthermore, the gates in protein ion channels are also believed to be in the same order of magnitude as the sizes of lipid pores. The gate of the voltage-gated potassium ion channel Kv1.2 was reported to be of the order of $0.45 - 0.5nm$ (*Treptow and Tarek*, 2006), and the structure of the acetylcholine receptor channels revealed a radius of $\sim 0.3nm$ (*Corry*, 2006). The Pauli radii of K^+ and Cl^- ions are $0.131nm$ and $0.181nm$ (*Vieira*, 2002), respectively, from which it can be assumed that, even with the additional diameter of water molecule $2.72\mathring{A}$, both the lipid pores and the ion protein structures reported in the literature are big enough to let these ions through.

For comparison, hydrophobic pores formed in the lipid bilayer, referred to as 'water-filled defects' were reported to have a radius 0.3 to 0.5 nm (*Antonov et al.*, 2005). Under sufficiently large electrical fields they are believed to extend to hydrophilic pores, by reorientation of the lipid molecules, and be of an effective radius of 0.6 up to 1 nm (*Glaser et al.*, 1988).
It needs to be noted that the electrolyte-filled pore model does not take the boundary conditions at the pore wall into account. Some studies show that the boundary potential might influence single channel conductances (*Bell and Miller*, 1984), which could then influence the mean pore size.
However, one should also consider the structured water lining the pore walls and dielectric breakdowns of the bilayer as a possible explanation for observable quantised current events. In this case the liquid-filled pore model would no longer be applicable in determining the mean pore size.

On the example of DPPC membrane, it was shown that exposure of BLM to temperatures in the vicinity of the main phase transition temperature causes a detectable change in membrane capacitance. This is believed to be partially attributable to a thinning of the lipid membrane due to its vicinity to the melting transition and partially to the solvent wash-out from the membrane (discussions with V.Antonov and (*Antonov et al.*, 2003)).

If the increase in the membrane capacitance with an increase of temperature towards the lipid melting transition was only due to the decrease in membrane thickness, the DPPC membrane would need to become thinner by $\sim 48\%$

(*fig.*37). Earlier observations by *Gennis* (1988) or *Antonov et al.* (2003), following the same approach, reported a membrane thickness change of 17% and 65%, respectively.
There is no doubt however that the thinning of the bilayer is only one contribution in the maximum membrane capacitance at the lipid melting temparature. It is believed that the redistribution of the organic solvent might also cause a change in the membrane dielectric constant (*Antonov et al.*, 2003). Some other possible explanations are also reported in the literature. Gennis, for instance, in (*Gennis*, 1988) proposed that the thinning of the BLM might be due to large changes in pressure between the water layers on both sides of the bilayer, which in turn causes redistribution of the solvent in the bilayer. It was also observed that the lifetimes of channels in BLM could be related to the amount of solvent contained in the bilayer (*Rudnev et al.*, 1981) and the actual amount of the solvent in the membrane can be deduced from the dependence of capacitance on the applied voltage (*Toyama et al.*, 1991). The question of solvent present in the membrane needs to be addressed in future research more thoroughly and the capacitance measurements might be the key to start the approach. It should be noted, that single BLM experiments succeeded in membrane formation without the presence of solvent, which suggests that hydrocarbon solvent need not be present. The determination of the maximum membrane capacitance at the melting transition temperature could also serve an an interesting subject for future research.
It should also be noted, that if one takes the ordered water in the pore as a likely scenario, the dielectric properties of the membrane would also be changed and the equation relating the membrane capacitance to membrane thickness $C = \varepsilon_0 \varepsilon_r \frac{A}{d}$ would no longer be apply.

Protonic transitions

Preliminary experiments invesigating the effect of pH on durrent fluctuations in the membrane confirmed the observations made by (*Kaufmann and Silman*, a,b, 1983) that proton concentration could introduce a membrane into 'protonic transition' and thus induce step-wise current fluctuations. This is a consequence of the fact that the chemical potential of the membrane changes with an increase in the number of protons in the system, in a similar manner as with proteins or anaesthetics in the membrane.

Consequently, if the lipids comprising the membrane are close to the melting transition regime, then the change in proton concentration in the membrane could change the thermodynamic state of the membrane such that it enters the melting transition. This change in chemical potential was seen as a modulation of current fluctuations for membranes made of DLPC:DMPC lipid mixture. In-

creasing the proton concentration on one side of the DPPC membrane moreover resulted in observable step-like current changes across the membrane. Since the melting temperature of DPPC is $\sim 41°C$ the current fluctuation could be interpreted as indicative of proton transport through the membrane rather than the result of the membrane melting.

Anaesthesia

The presence of octanol and ethanol in the DOPC:DPPC membrane caused a pronounced decrease in membrane permeability, modulating the current fluctuations in a concentration-dependent manner. This could be reasoned from the shift of melting profiles of lipids to lower values when the octanol/ethanol was added to the membrane system. Similar studies by *Blicher* (2007) further supported the observation that the change in membrane permeability was due to the resultant shift in melting transition of the membrane lipids. The increased permeation rate observed with the presence of octanol in the membrane observed by *Blicher* (2007) could be explained by the fact that the initial observation was made at temperature slightly below the main transition of lipid, and octanol and by moving the melting transition to lower temperatures at the same time will have increased the probability of pore formation in the membrane. In the set of BLM experiments reported here, the averaged membrane permeability decreased substantially from around $8.5 \cdot 10^{8 ions}/_{s}$ for pure lipid membrane clamped at 220mV to $2.5 \cdot 10^{8 ions}/_{s}$ with 7.9 mol% of octanol in the membrane and down to $4.35 \cdot 10^{7 ions}/_{s}$ and $2.4 \cdot 10^{7 ions}/_{s}$ with 15.9mol% and 23.8mol% of octanol, respectively. Small concentrations of ethanol (1-2mM) were also noted to have an inhibitory effect on lipid current fluctuations. Here however, the mean opening times of the channels were more affected than the mean current amplitudes. Ethanol seemed to stabilize the opening state for pores and the mean opening time was prolonged from arounf $4ms$ for pure lipid membrane to $\sim 12ms$ and $\sim 18ms$ for 9.5mol% and 19.5mol%, accordingly. Zuo et al. have inversigated the effects of octanol and ethanol on the acetylcholine receptor channels activity (*Zuo et al.*, 2004a,b, 2001). They arrived at the conclusion that octanol decreases the mean current amplitude of the channels whereas the ethanol favours the channel open state with a little influence on the channel conductance. The similarity of the findings with lipid ion channels is striking.

Anaesthetic effects of alcohols on proteins - what do we know?

Ethanol - a simple twocarbon molecule can produce many neurological and physiolgical effects. It is the least potent of all the drugs - concentrations of the order of 10-20mM are required to produce intoxication in humans and around 100-200mM to produce anaesthesia in experimental animals (*Fang et al.*, 1997).

Nevertheless, the nonspecific and nonreceptor mediated actions of ethanol were ignored in the light of recent findings that protein ion channels act as targets for alcohol. Identification of the specific protein sites responsible for the action of alcohol remain elusive, though.

Since acetylcholine receptor channels (AChRs) are known to be an important target for various chemicals, including nicotine, carbachol, d-tubocurarine, alcohols, general anaesthetics, some insecticides, heavy metals and several natural toxins (*Castro and Albuquerque*, 1993; *Ishihara et al.*, 1995; *Mori et al.*, 2001; *Nagata et al.*, 1997, 1998, 1996b; *Swanson and Albuquerque*, 1992), the fact that the channel activity in the presence of any of them is changed should not be surprising. Understanding of the detailed mechanism of the action of anaesthetics (and other factors) is essential in controlling the release of multiple neurotransmitters at the synaptic level, in which the receptor is believed to play an important role. Hovewer, so far, little is known as to how alcohols affect AChR ate the single-channel level. Kinetic models suggest that the inhibitory action of alcohols on protein ion channels are the result of anaesthetic-induced changes in channel gating mechanism. Nevertheless, no proof of any such hypothesis is known so far.

Ethanol consumption is also known to modulate some μ- and δ-opioid receptors (*Froehlich et al.*, 1998; *Hyytiae*, 1993), as shown by the fact that their antagonists, such as naltrexone, can be used to treat alcoholism (*O'Malley et al.*, 1992; *Krystal et al.*, 2001) i.e., affect these receptors and thus diminish the effect of alcohol. Intracellular effects of opioid receptor activation are believed to include, among many others, increased potassium channel conductance (*Christie and North*, 1988; *North et al.*, 1987) and decreased calcium conductance (*Rhim and Miller*, 1994).

Last, but not the least, the activity of the gamma-aminobutyric acid (GABA) receptor was also found to be modulated by a variety of different compounds, including benzodiazepines, barbiturates, neuroactive steroids (*Akk et al.*, 2004) and ethanol (*Akk and Steinbach*, 2003). In some studies the potentiating influence of ethanol on GABA receptors was revealed by increasing channel mean open time in the presence of ethanol (*Akk and Steinbach*, 2003; *Celentano et al.*, 1988; *Harris et al.*, 1995), whereas no other significant effect of ethanol on GABA receptor activity was reported (*White et al.*, 1990; *Zhai et al.*, 1998). Even though the influence of ethanol on GABA receptors has remained unclear (*Aguayo et al.*, 2002), the idea that ethanol interacts directly with specific sites on ion channels has remained unquestioned. The lack of action of ethanol on some GABA receptors was explained by 'heterogeneity and differences in intracellular modulation by protein kinases and calcium' in these neurons (*Aguayo et al.*, 2002). Studies to identify the channels responsible for alcohol-induced

behaviour are now being conducted.

There is considerable controversy over whether the process of anaesthesia occurs by direct binding of anaesthetics to proteins or secondarily, by nonspecific perturbation of lipids. It is generally agreed that anaesthetics act ultimately on neuronal ion channels, even though the nature of the binding sites remains unclear for the present state of knowledge. Although the mechanisms of various anaesthetic agents are poorly understood at a molecular level, general anaesthesia is widely employed in general practice nowadays. Every year 9mln patients undergo anaesthesia in France (*Clergue et al.*, 2002) and more than 21 mln of the population receive general anaesthesia in North America (*Beverley*, 2008).

The inhibitory action of most anaesthetic compounds on ion channel currents measured by patch-clamp techniques seems to suggest a protein-targetted anaesthesia approach and for many it is considered to be evidence for proteins as the main anaesthetic targets. However, already in the field of protein ion channels a lot of contradictory information exists about how the anaesthetics might modulate the electrophysiological response from protein ion channels. The best known, inhibitory action, is also thought to be applicable to the inert gas xenon and nitrous oxide (*Yamakura and Harris*, 2000). This behaviour on the other hand turned out to be only partially true for cyclothiazide, which was found to inhibit currents from GABA-associated receptors and yet activate those of glutamate receptors, therefore exhibiting the opposite effects of 'normal anaesthetics' (*Deng and Chen*, 2003). Chlorpamazine, an antipsychic drug has, on the other hand been found to alter membrane biomechanics and leave the membrane conductance intact (*Lue et al.*, 2001).

Lipid-mediated anaesthesia

There are already some reports supporting the hypothesis of a lipid membrane-mediated action of anaesthetics. *Leonenko and Cramb* (2004) have observed ethanol induced changes in thickness of BLM bilayers and proposed that the presence of ethanol could promote the phase change of the lipid. Similar conclusions were drawn by *Mitchell et al.* (1996) about lipid-mediated action of alcohol on rhodopsin and other G protein-coupled receptors. *Fa et al.* (2006) pointed to the the action of the antibiotic azithromycin on the melting behaviour of DOPC and DPPC membranes and aspirin was found to disrupt membrane stability by decreasing membrane stiffness and membrane thickness by *Zhou and Raphael* (2005). Furthermore, any structural changes in the membrane induced by anaesthetics might also affect the function of the membrane embedded proteins that act as ion channels.

In addition to the theory that anaesthetics exert their actions by changing the heat capacity profiles of membrane lipids (*Heimburg and Jackson*, 2007, 2006; *Seeger et al.*, 2007), the literature also suggests other means by which lipid-mediated anaesthesia may work. Robert Cantor, for instance, suggests that changes in the lateral pressure of membrane in the presence of anaesthetics may alter the conformational equilibrium of membrane-embedded protein ion channels and thus induce the effect of anaesthesia (*Cantor*, 1997). Roth et al. propose that the anaesthetic action can be understood by the actions channels' hydrophobic gates, referred to as bubbles (*Roth et al.*, 2008).

Ion channels and anaesthesia - what is the future outlook?

It is believed that the results presented in this thesis could introduce a seed of change in the approach to finding the answers to the questions surrounding ion channels and the phenomenon of anaesthesia. The process of lipid phase transition has been shown to be relevant also in biological systems (*Heimburg and Jackson*, 2005, etc.). The only difficulty is that due to the complexity of a biological membrane the lipid transitions alone are hard to distinguish from temperature-dependent protein conformation changes. It is however reasonable to assume that there might be a correlation between lipid related processes and the scales of protein ion channel transitions. These conformational changes give rise to channel opening and closing, which happen in range of $1-100ms$, similar to the lipid relaxation times. This is also the time regime of action potentials.

The lipid melting transitions, among many other characteristics, make lipids a very important component of a membrane, which cannot be left out in the considerations about ion channels and anaesthesia.

The simple lipid systems used so far could be a basis for investigations of more complex systems, including proteins. Even more interesting conclusions might be drawn by adjusting external factors such as temperature, voltage or varying the anaesthetics content in the membrane. Thermodynamics offers a suitable method of choice to address these questions as it shifts the focus to assemblis of molecules rather than considering the problem at a single-molecule level. It provides the analytical the tools which can serve as a frame of reference in explaining the observed phenomena and gives the room for the optimization of a number of physical parameters.

The results presented in this thesis might moreover pose some questions about the reliability and consistency of ion channel data currently available (see fig.). At the phase transition, lipids undoubtedly possess properties that cause them to exhibit behaviour very similar to the electrophysiological activity of a protein ion channel. At the same time however, single channel currents

were also observed for some non-biological sources such as a rubber surface or polyethylene filters (fig.). This highlights the fact that there is still an enormous number of questions that need to be answered in the field. Investigation of the concept of ordered water might provide a suitable first step in following up the work (*Higgins et al.*, 2006; *Das and Singhal*, 1985)

We live in the era of ion channels. Every question concerning the mechanism of the action of ligands with the cells is being answered by finding an appropriate receptor membrane protein, which in many cases works as an ion conducting channel. Very recently we have learnt about channels controlling the sense of smell, channels participating in the process of photosynthesis in plants or acid sensing ion channels. In the light of large families of voltage-gated protein ion channels and ligand-, odour- acid -, bubble - gated ones, could therefore lipid ion channels be given a name of the temperature-gated ion channels?

"If you would be a real seeker after truth, it is necessary that at least once in your life you doubt, as far as possible, all things."

Rene Descartes (1596-1650)

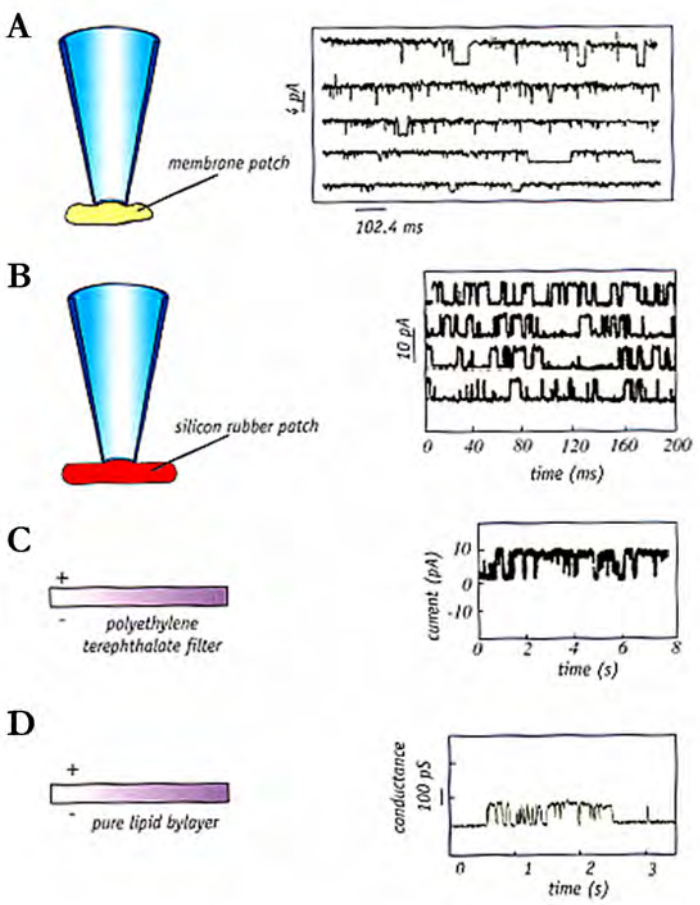


Figure 54: 'Single channel' currents recorded from non-biological sources should make many of us pause to reflect on reliability of protein ion channel data reported in literature. *A*-recordings from anion-selective ion channels from human epithelial cells (*Tabcharani et al.*, 1989), *B*-ion channel-like currents from patch clamp on rubber surface (*Sachs and Qin*, 1993), *C*- discrete fluctuations from synthetic filters of polyethylene (*Lev et al.*, 1993) and *D*- currents which followed injection of lipid vesicles near membrane(*Woodbury*, 1989). Figure adapted from (*Pollack*, 2001).

Figure 55: The nematode Chaenorabditis elegans consists of 10^3cells, whereas its genome contains genes for > 230 different ion channels. The human body consists of $10^{13} - 10^{14}$ cells and no finite number of protein ion channels encoded by genes are yet been found. The lipid matrix has proven again that it is not to be left out in the considerations of such aspects as membrane permeability nor the action of anaesthesia. Many questions are still longing for an answer.

7 Appendix

Voltage vs. electroporation

The influence of voltage on pore fomation process has been investigated pararelly with the effect of temperature, in particular with respect to the range of melting temperatures of lipids under consideration. The effect of membrane electropermeabilization was excluded from the considerations below, due to the non-electroporative voltages ($< 500mV$) applied (*Chizmadzhev and Weaver*, 1996). *Bhaumik et al.* (1983) and *Antonov et al.* (1990) were the first to postulate that melting transition of phospholipid membranes might be electric-field dependent, such that lipid packing density changes under the influence of external electric fields.

The idea might be pictured as if electrical field had an ordering effect on lipid polar head groups and hydrophobic chains relative orientation (see *fig.*53 and also (*Stulen*, 1981; *Cotterill*, 1978; *Saux et al.*, 2001; *Berestovsky et al.*, 1978; *Dimitrov*, 1984)). By this means, the external electric field could induce a melting effect in lipid monolayers if they are initially close to their melting temperature conditions. It is of great interest to explore the problem more thoroughly and piece all the information together.

BLMs from DPhPC and DPPC

Diphytanoyl derivatives of phosphatidylcholine (DPhPC) are believed to be useful in "planer lipid membrane" research. Their heat capacity profiles do not show significant peaks, which does not imply specifically in which temperature regions the probability of pore formation would be the highest. This is also a reason why the DPhPC membranes are relatively stable under the BLM conditions. Nevertheless, current fluctuations were observed in membranes made from DPhPC at room temperature conditions. The voltage triggered the poration of the membrane: the higher voltage the bigger were amplitudes of the fluctuations imitating pore opening and more subcondactance steps were present. The effect was described by relating the stedy-state open probability derived through dwell-time analysis with the value of voltage applied.

Similar approach, on how the voltage can induce the membrane poration, was investigated on membranes made from DPPC - a lipid which shows pronounced peak in a heat capacity profile at 41,5°C, i.e. at the temperature quite distant from the temperature at which the experiments were performed (room temperature, $\sim 21.5°C$). From *fig.*58 it can also be seen that the heat capacity value of the peak for DPPC is three oder of magnitude higher than that of DPhPC.

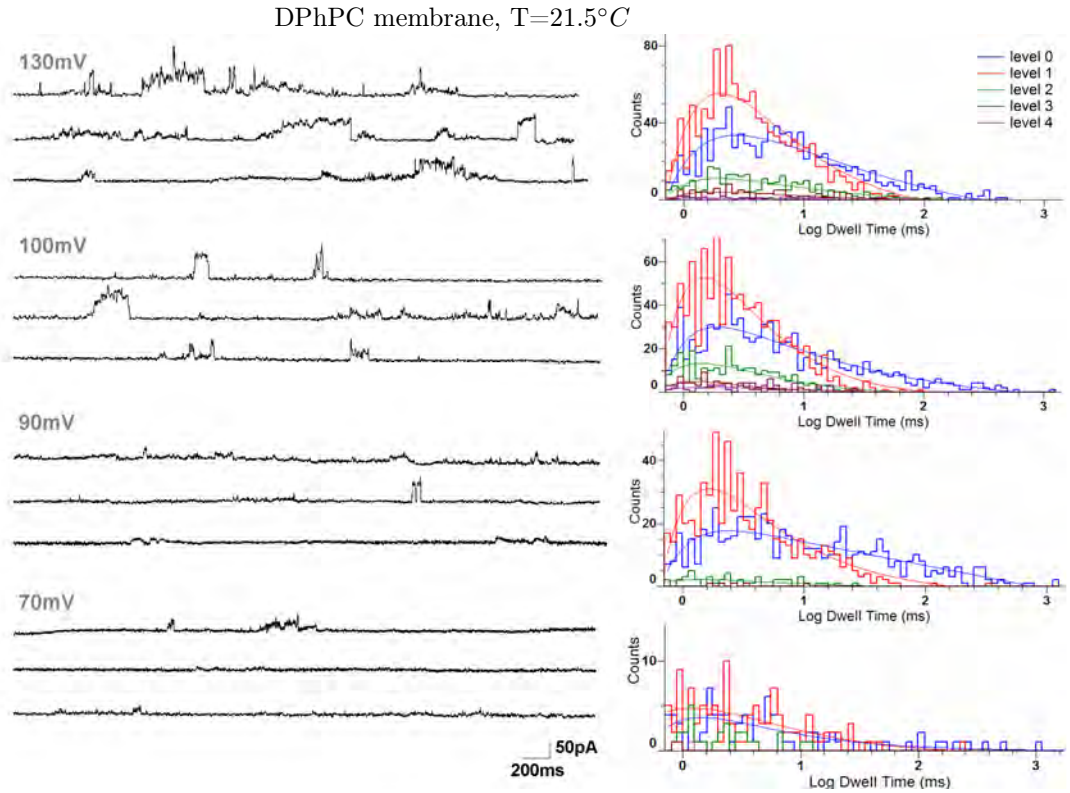


Figure 56: Gating and permeation of DPhPC lipid ion channels. One can note that the current flucuations, resembling opening and closing of a channels, are not perfectly rectangular in shape. Different effect is observed when the membrane is in its melting transition and the current fluctuations are not solely the effect of transmembrane voltage (see *section Results*). No pores were observed at 60mV and lower voltages, from which it is tempting to conclude about the pore formation process as a 'threshold' phenomenon. The DPhPC membrane was observed to break at voltages of the order of $200mV$.

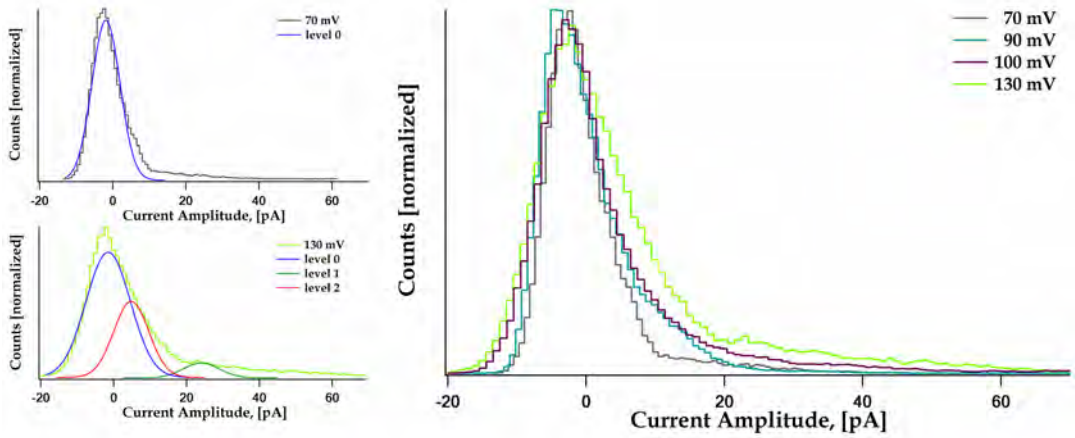


Figure 57: Amplitude histograms for DPhPC membranes. Higher transmembrane voltage causes more current fluctuations to occur and as a result histogram broadens.

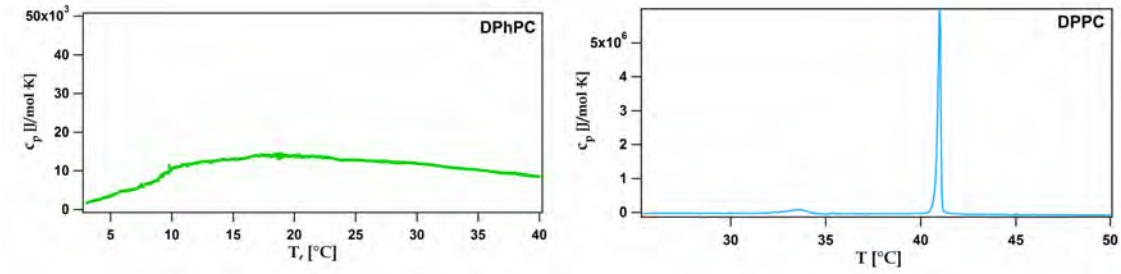


Figure 58: Heat capacity profiles of DPhPC and DPPC. Melting profile of DPhPC shows no pronounced peak, which would indicate that the main melting transition happens over a wide range of temperatures. The values of the excess heat capacity are also significantly lower compared to DPPC maleing transition, which can point to the lower probability of pore formation in the membrane.

Influence of transmembrane volatge on pore formation in DPPC membrane was similarly investigated. Usually however, the voltage at which the current fluctuations started to be observed was in the range of 100-120mV, i.e. about 50mV more than in the case of DPhPC. It can be assumed that the difference in the voltage needed to porate the membrane lies in the offset in the excess heat capacity value in DPhPC compared to DPPC (*fig.*58). The non-zero heat capacity value for DPhPC at the temperature of the measurements $\sim 21.5°C$ might suggest a certain probability for the occurrence of pores at the temperature of measurements, unlike in DPPC membrane, where the heat capacity has only non-zero value at its pre- and main melting transition.

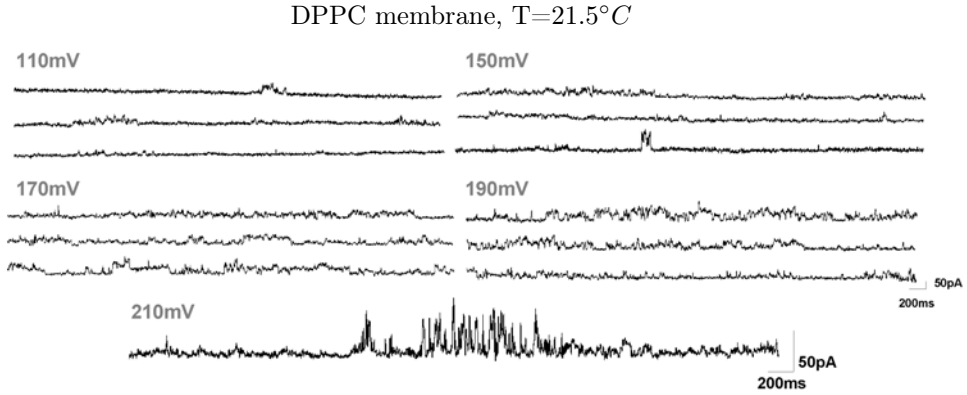


Figure 59: Influence of voltage on pore formation in DPPC membranes.

From dwell-time analysis for DPPC system, the probabilities of opening of all levels have been derived and the steady-state open probability was plotted as a function of voltage. The steady state open probability measured as a function of test potential is a popular way of characterising the voltage dependence of gating of protein ion-channels (see*fig.*62*C*). The higher probability for the lipid channels to open at higher voltages might also suggest that voltage is the factor that can introduce lipid into melting transition.
In an attempt to predict the voltage influence on heat capacity of the lipid melting, a simple qualitative analysis has been perfomed. It has been observed that, the two systems require different voltages applied in order for the open probabilities to be similar in the two cases. This can, sunsequently, be explained by the fact that at the temparature of the experiments ($21.5°C$) the DPhPC membrane could already be characterized by the non-zero excess heat capacity value, which predisposed the pore forming in the membrane. Therefore, the lower voltage applied to DPhPC membrane was usually enough to induce the pores in the membrane of the same opening likelihood as in the case of DPPC membrane, which required higher voltage (*fig.*60).

From the analysis in *fig.*60 it could be concluded that the voltage of $60-70mV$ is needed to make up for the heat capacity difference of $14kJ/molK$ and, possibly to induce the poration of the membrane. It is not to be considered as a general statement, but only a starting point to investigate the problem more carefully in the future. It is worth mentioning that the effects of temperature and the voltage on the lipid pore formation cannot be studied separately, i.e. in the course of the experiments it was very rare to observe the channel-like current fluctuations at $0mV$ voltage even if the membrane was examined at the exact temparature of the phase transition of the membrane lipids.

	$DPhPC$	$DPPC$
$P_{open}[\%]$	17.4	13.3
$V[mV]$	90	150
$P_{open}[\%]$	60.7	55
$V[mV]$	100	170

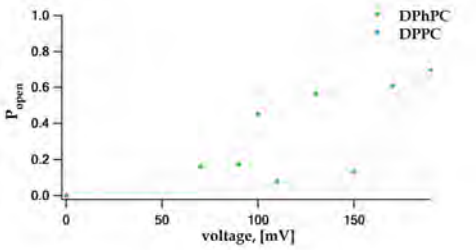


Figure 60: Heat capacity vs. voltage analysis on the example of two signle lipid systems: DPPC and DPhPC. Steady state open probability is higher at the higher voltages across the membrane.

Table 1 ION CONCENTRATIONS AND EQUILIBRIUM POTENTIALS FOR A TYPICAL NEURON

Ion	$[Ion]_{out}$ (mM)	$[Ion]_{in}$ (mM)	$[Ion]_{out}/[Ion]_{in}$ (mM)	E_{rev} (mV)
Ca^{2+}	2	$<10^{-4}$	>20,000	> +132
Na^{+}	140	12	12	+66
K^{+}	4.2	140	0.03	−94
Cl^{-}	115	40	2.9	−29

Figure 61: Ion concentration and equlibrium potentaisl for a typical neuron *Cannon* (2003).

Protein ion channels by numbers

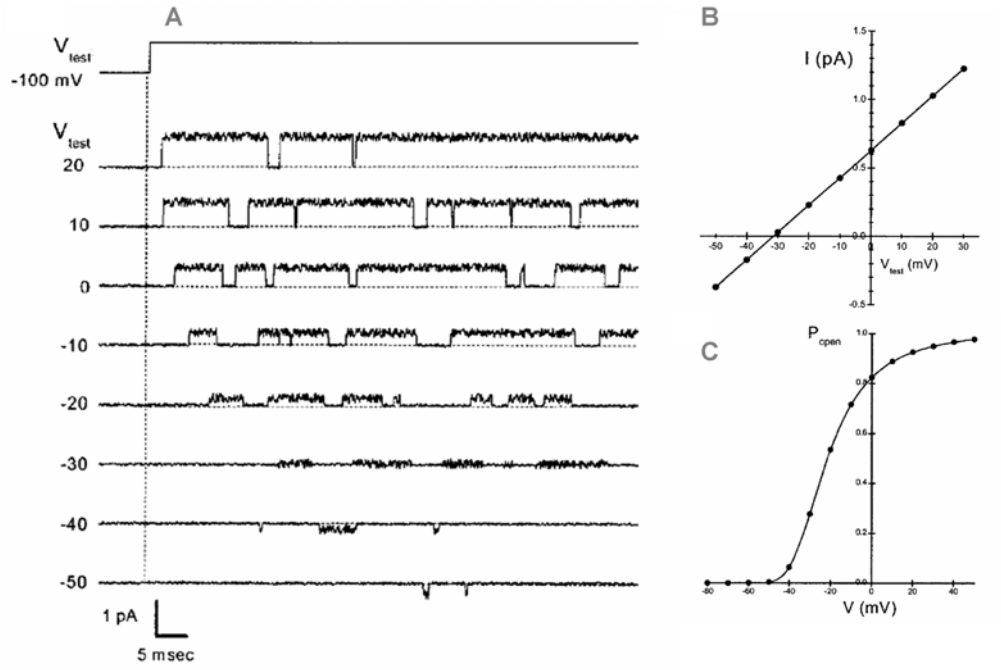


Figure 62: Gating and permation of single potassium ion channels. *A*- the channel activates upon membrane depolarisation and its permeability is characterised by a current-voltage relation (*B*). The voltage dependence of gating can moreover be characterised by the steady-state open probability of the channel measured as a function of the electric potential (*C*). Picture adapted from *Cannon* (2003)

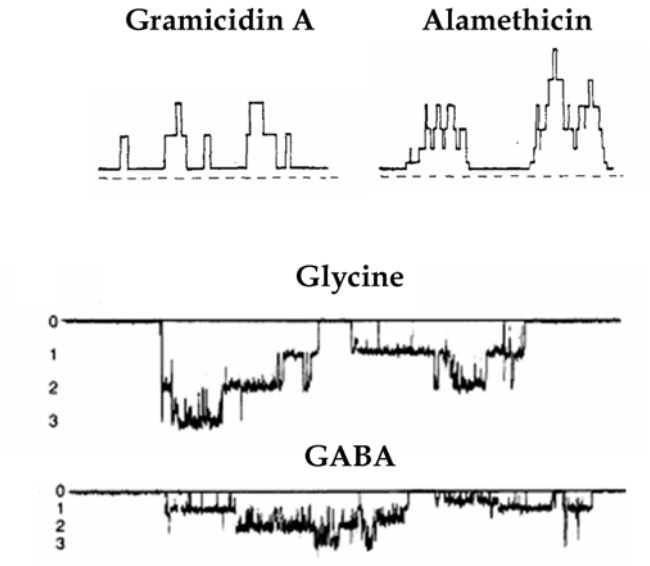


Figure 63: Multilevel conductance states for Gramicidin A channels, alamethicin channels (*top*, *and E Neher and Stevens* (2003)) and Glycine and GABA receptor channels (*bottom* (*Sakmann*, 1992)). In all cases multilevel conductance states can be seen. They are usually attributed to different conformational states of protein channel when undergoing open and close transitions.

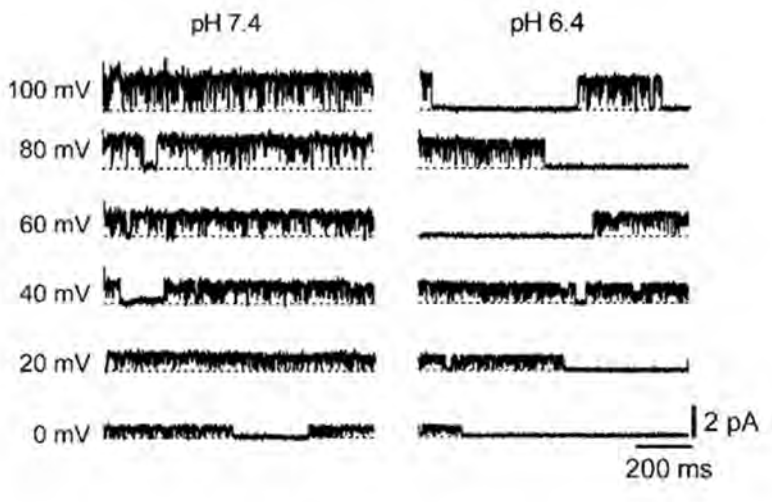


Figure 64: Potassium ion channel macroscopic currents were found to be sensitive to extracellular pH change (*Kwan et al.*, 2006).

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