**Original Paper**

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2004;14:231-240

Accepted: June 17, 2004

# Membrane Thickness Changes Ion-Selectivity of Channel-Proteins

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**Key Words**

ICln • Volume regulation • Bilayer • Reconstitution • DLPC •DMPC • DPPC • Diph-PC • DSPC • DAPC

**Abstract**

The plasma membrane is a highly dynamic cell-barrier if the nature and distribution of its constituents are considered. Ion channels are embedded in these double lipid bilayers, which modulate their 3D-structures. The structure modulations by the lipid bilayer can assume such a degree that channel activation depends on them, as was shown for the KcsA potassium channel. Here we show that the cation-over-anion selectivity of reconstituted ICln channels can be varied by the thickness of a bilayer build of phosphatidylcholines. The shorter the acyl-chains and therefore the thinner the bilayers of the membrane are, the more potassium selective the channels are. In contrast, the longer the acyl-chains and therefore the thicker the membranes are, the more chloride selective the channels become.



## Introduction

Ion channels accomplish their function of ion-permeation after being inserted into the lipid double-layer of the membrane [1]. The process of insertion is poorly understood and most likely rather heterogeneous. It is reasonably well established that the 3-dimensional (3D)-structure of the lipid-immersed channel protein deviates from the structure of the protein dissolved in water [2-6]. Once the ion channel is placed into the fluid double-lipid-layer of the membrane, it would be comforting to believe that the channel proteins assume an energetic minimum, leading to a stable structure. However, molecular insights into the functioning ion channels indicate that the processes of ligand-binding [7] and/or voltage sensing [8] are able to modify the 3D-structure of the pore-protein. Moreover, the 'dynamic' global energetic minimum of the channel proteins depends on the lipid environment in which they are immersed [9], as it was shown for the KcsA potassium channel. The activity of this channel depends upon the presence of negatively charged lipids [10]. The lipid composition of the plasma membrane, i.e. cholesterol and the different characteristic phospholipids - solely to mention those two – varies

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considerably from cell to cell in the same species and also within the same type of cells within different species. This complexity is topped by the fact that even the inner and outer leaflets of the plasma membrane have different lipid compositions [4, 9, 11, 12], which can, however, be mixed by phospholipid-translocases also known as flippases [13, 14]. In order to gain more insight into this highly complex system of 'lipid neighbourhood-depending channel function', we set out to study the influence of the acyl-chain length of phosphatidylcholines on the selectivity of the reconstituted ICln channel protein. This channel protein seems particularly suitable for these kinds of experiments, since it was shown that the pK/pCl value of reconstituted ICln in a Diph-PC bilayer was changed after reconstituting the channel protein in heart-lipid-extract bilayers [15]. The reconstitution in the Diph-PC membrane leads to channels, which are four times more permeable for potassium than for chloride, and must therefore be considered as potassium-channels. However, if the same channel protein is reconstituted in a mix of native lipids isolated from heart cells, the channels change their selectivity from potassium to chloride [15]. This behaviour led us to speculate that the lipid environment could substantially influence the structure of the ion permeating pores. Since the native lipid extract isolated from heart cells that were used in the experiments is a mixture not only of known (and therefore specified in their percentage-weight) but also of unknown components, we set out to test how different synthetic (and therefore unambiguously chemically defined) lipids can influence the ion selectivity of a pore forming protein. Here we describe the influence of phosphatidylcholines with different saturated acyl-chain lengths varying from (C12:0) to (C20:0) on the cation-over-anion selectivity of reconstituted ICln channels.

## Materials and Methods

*Purification of the ICln protein:*

The open reading frame (ORF) of ICln was cloned in frame into the pET3-His vector [16], adding a histidine tag (H-tag) to the N-terminus of ICln. In a previous set of experiments we assessed the effect of the H-tag attached to the C-terminus and found no difference compared to the N-terminal H-tag [16]. The H-tag allows purification of ICln on a Ni-NTA agarose column (Qiagen, Germany). A single protein band of the expected size can be obtained after the over expression and subsequent purification of ICln in *Escherichia coli* (BL21 (DE3)). The purified ICln protein was stored at –74°C in an elution buffer (50 mM $K_2HPO_4$ and 200 mM imidazole, pH 8.00) at a concentration of ~ 0.4 µg/µl.

*Bilayer experiments:*

*A) Macroscopic currents.* The experimental procedure used for the reconstitution of ICln in 'black' lipid bilayer experiments was previously described in detail [15, 16]. The lipid bilayer was painted on an aperture of 1 mm diameter in a Teflon diaphragm separating the *cis* and *trans* compartments of a recording chamber, each holding 5 ml aqueous solution. For the lipid bilayer membrane 1% (w/v) lipids in n-decane and butanol were employed. The lipids were used as the sole component for the bilayer membrane, or in a mixture with Diph-PC as indicated. After the membrane had turned optically black in the reflected light and its stability was assessed, the protein (500 ng/ml) was added to the *cis* and *trans* chambers. The experiments were made at 28°C. The membrane current was measured by a pair of Ag/AgCl reference electrodes (Metrohm, Switzerland) connected in series with a voltage source (*cis*) and a 'current to voltage converter' (*trans*), which was made using a Burr Brown operational amplifier (9407/0541F). The signal was recorded with a strip chart recorder (BBC, Austria). The plain membranes remained stable for more than an hour (tested in the absence of the ICln protein).

*B) Selectivity tests.* In order to determine the ion selectivity of reconstituted ICln, we chose a gradient for the ions employed that had a higher salt concentration in the *trans* chamber (150 mM KCl, 5 mM HEPES, pH 8.00), and a lower concentration in the *cis* chamber (10 mM KCl, 5 mM HEPES pH 8.00). Since the establishment of the bilayer membrane requires time – during this time the concentration gradient becomes reduced in both chambers communicating through the circular hole (area ~ 1 $mm^2$) – the chloride gradient ($\Delta PD_{Cl}$) was measured after each experiment, using chloride-selective electrodes before the membrane was disrupted. The $\Delta PD_{Cl}$ value of every single experiment was used to calculate the pK/pCl value according to the Goldman-Hodgkin-Katz (GHK) equation. The respective reversal potentials were determined graphically by interpolation and were normalized to a gradient of 15 [16].

*C) Tip-dip experiments (single channel measurements).* The planar lipid bilayers were established on the tip of patch pipettes having a resistance of 10-20 MΩ in a 100 mM KCl solution as described [16, 17]. The lipids were spread on the surface of the recording chamber electrolyte solution and the tip of the pipette was passed at least two times through the lipid monolayer forming a seal whose resistance was 20-50 GΩ. Single channel measurements were made with all lipids used for the macroscopic current measurements (see above), except for the mixtures DiphPC/DSPC 1:4 and DiphPC/DAPC 1:1, since it was technically impossible for those lipids to obtain membranes on the patch pipettes, most likely for the high percentage of long acyl-chains used for forming the membranes. The salt solutions used were: pipette (100 or 10 mM KCl, 5 mM HEPES, pH 8.00 or pH 6.00 as given in the text), and bath (100 mM KCl, 5 mM HEPES, pH 8.00 or pH 6.00 as given in the text). The protein was added only to the bath solution after the development of a stable seal. Single-channel currents were measured using a patch-clamp amplifier and the data were saved to the hard disk. For the standard analysis the data were filtered at 0.2 kHz.

*Atomic Force Microscope (AFM) measurments*

*A) Preparation of supported lipid bilayers.* Vesicles were prepared from DiphPC alone or by mixing Diph-PC and DAPC 1:1 from 2% chloroform stock solutions. The chloroform was evaporated under a stream of nitrogen gas and the lipid mixtures were rehydrated overnight in Milli-Q™ Ultrapure Water (Millipore) to yield a final concentration of 2 mg/ml. The lipid mixture was vortexed for 3 min to produce multilamellar vesicles and subsequently sonicated for 30 min at 50°C (Decon Laboratories, Hove, UK) to produce small unilamellar vesicles. 10 µl of the vesicle solution were placed on freshly cleaved mica (Goodfellow, UK) and allowed to adhere for 1 min at room temperature (22°C). Subsequently 50 µl of HBS (150 mM KCl, 5 mM Hepes, pH 8.0) were added for 3 min and the samples were then rinsed 3 times with Milli-Q™ water, dried under a stream of nitrogen gas and transferred to the AFM.

*B) Atomic Force Microscopy (AFM).* AFM imaging was performed with a Multimode Atomic Force Microscope (VEECO Instruments Inc., Santa Barbara, CA) equipped with a J-scanner and with an in-line electronics extender module and controlled by a Nanoscope IIIa controller. The microscope was placed on a silicone-gel vibration isolation pad and a pneumatic table and placed under an acoustic hood to reduce ambient room sound interferences. All experiments were performed at room temperature (22°C). Imaging was performed in the air tapping mode with commercially available $n^+$-silicon sensors on cantilevers with a specified spring constant of 42 N/m (NCH-50 Pointprobes; Nanosensors™, Wetzlar-Blankenfeld, Germany) tuned at a drive frequency of ~300 kHz. The drive amplitude was adjusted to produce a root mean square amplitude of ~1.7 V. Force was minimized by adjusting the setpoint just below the jump-off point of the tip. The images were captured at a 512 x 512 pixel resolution and the scan rate was 1 Hz. Images were flattened and analyzed using the algorithms provided by the Nanoscope software.

*Salts, chemicals and drugs*

All salts and chemicals that were used were *p.a.* grade. The different lipids were purchased from Avanti Polar Lipid (USA).

*Statistical analysis*

All values are given as mean ± SEM. Data were tested for differences in the means by a Student's t-test after verifying that the individual data points are normally distributed by using the Kolmogorov-Smirnov-test. A statistically significant difference was assumed at $p<0.05$.

## Results

The ICln protein was identified by expression-cloning using a cDNA library made from Madin-Darby-canine kidney cells (MDCK) [18]. In order to test, whether or not, the protein itself can form ion channels we reconstituted ICln in artificial lipid bilayers [16]. As a membrane we used 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Diph-PC) [15-17]. Since Diph-PC is able to form stable membranes, and phosphatidylcholines can be identified in many biological membranes, this lipid is frequently used for reconstituting channel proteins in artificial bilayers [19, 20].

Reconstituting the ICln protein in Diph-PC bilayers leads to a potassium selective current with a reversal potential ($E_{rev}$) of +33.12 ± 3.57 mV (n=9; figure 1), which corresponds to a pK/pCl of 5.14 ± 0.65 (n=9). The single channel conductance and open probability ($p_o$) of ICln channels in Diph-PC at +80mV are 2.55 ± 0.18 pS and 0.11 ± 0.06 (n=5; figure 2). These values are well in agreement with the values published earlier [15-17], obtained under the same experimental conditions. Diph-PC is comprised of two saturated side chains each of 16 carbon-atoms (C16:0[$(CH_3)_4$]) in length. In contrast to all of the other phosphatidylcholines used in this study, each of the two acyl-chains of Diph-PC contains four -$CH_3$ side-groups (total of eight *per* molecule). These side-groups seem to be important for the observed selectivity of reconstituted ICln, since the reconstitution of ICln in 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; a phosphatidylcholine with the same acyl-chain length of 16C, but without the methyl-groups) leads to a reversal potential ($E_{rev}$) of +45.53 ± 2.20 mV (n=6; figure 1), corresponding to a pK/pCl of 9.86 ± 1.31 (n=6). Both values are statistically significantly different from the values obtained using Diph-PC, indicating that ICln reconstituted in DPPC is more potassium selective compared to the reconstitution in Diph-PC membranes.

As aforementioned, Diph-PC is used as a result of the fact that the respective bilayer membranes are stable in order to allow current measurements for a period longer than an hour. Using DPPC the membranes are still stable (see above), however, for all of the other phosphatidylcholines we used, it was not possible to obtain stable membranes by using the respective pure lipids. Therefore, 1:1 w/w-mixtures of the different lipids with Diph-PC were used. For Diph-PC/DPPC, as shown in figure 1c, the mixture does not change significantly $E_{rev}$ (+47.03 ± 4.48 mV; n=6), pK/pCl (12.98 ± 3.53; n=6) nor the corresponding single channel conductance (1.80 ± 0.1 pS, n=2).

The reduction of the acyl-chain length to 14C (1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPC), or to 12C (1,2-dilauroyl-sn-glycero-3-phosphocholine; DLPC), respectively, does not lead to a further change of $E_{rev}$, or single channel conductance, if compared to DPPC (figure 2 and 3). The respective $E_{rev}$ and single channel

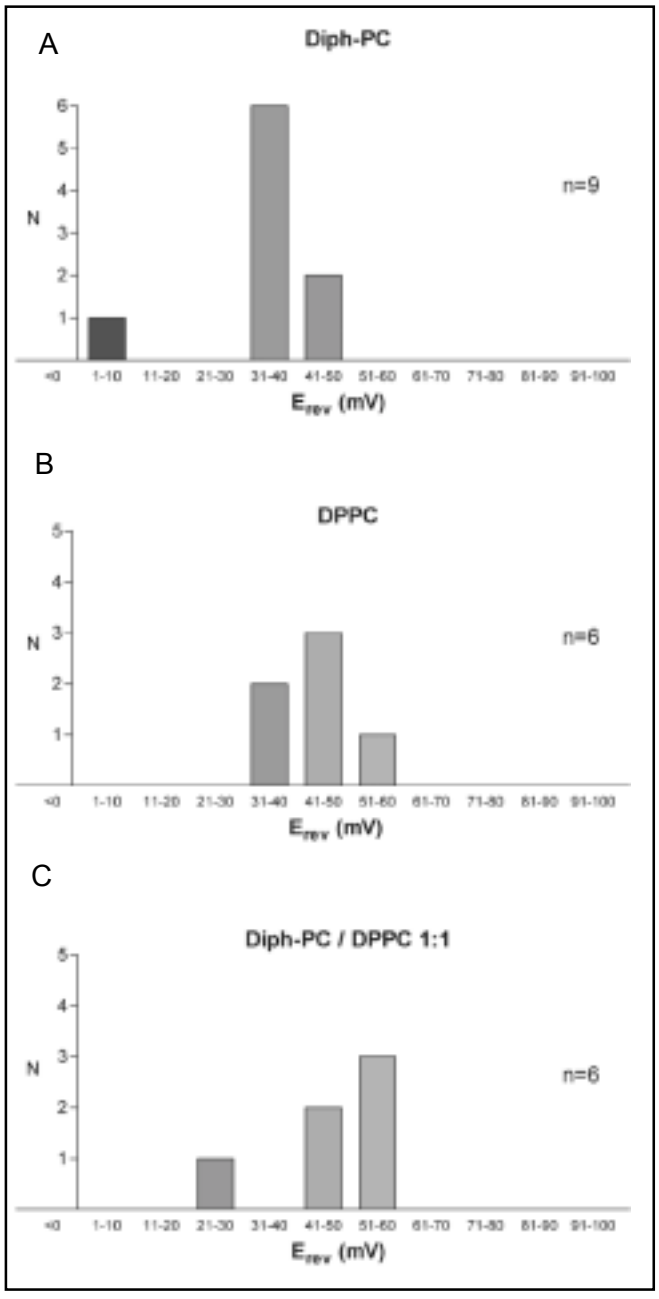
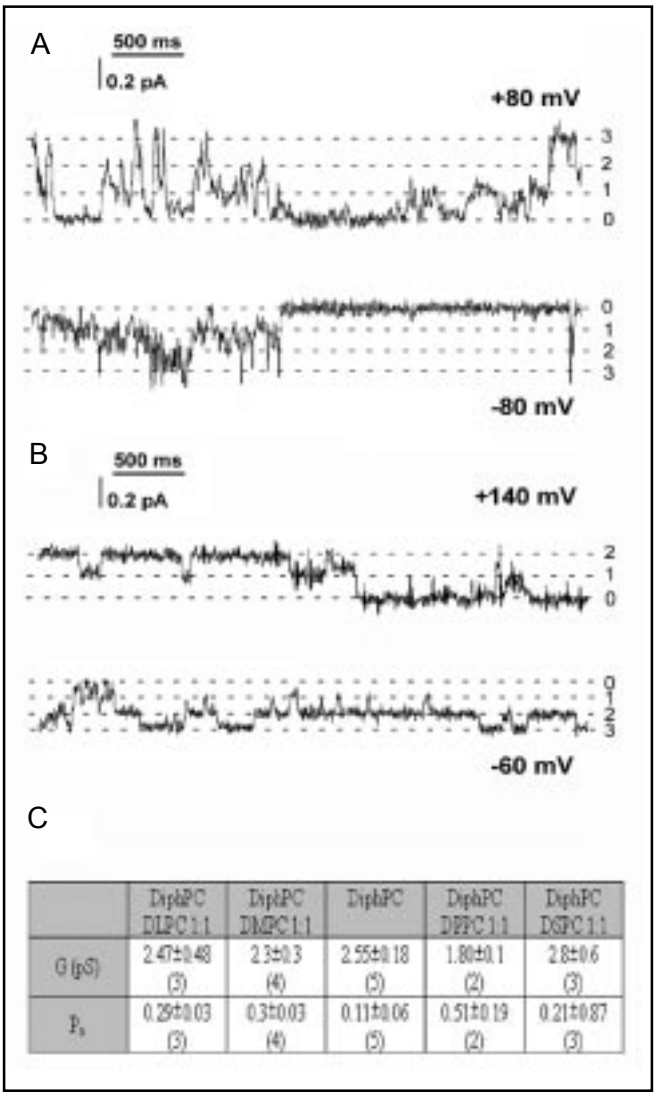



**Fig. 1.** Reversal potentials of the currents elicited by the reconstitution of ICln in artificial lipid bilayers. A) Reconstitution of ICln in Diph-PC. Positive $E_{rev}$ values indicate potassium selective currents. The higher $E_{rev}$, the more potassium selective the currents are. Negative $E_{rev}$ values (<0) indicate chloride selective currents. B) Reconstitution of ICln in DPPC. C) Reconstitution of ICln in a lipid mixture of equal parts (weight) of Diph-PC and DPPC (1:1). The individual $E_{rev}$'s are clustered in classes of 10 mV.




| | DiphPC DLPC 1:1 | DiphPC DMPC 1:1 | DiphPC | DiphPC DPPC 1:1 | DiphPC DSPC 1:1 |
|---|---|---|---|---|---|
| G (pS) | 2.47±0.48 (3) | 2.3±0.3 (4) | 2.55±0.18 (5) | 1.80±0.1 (2) | 2.8±0.6 (3) |
| $P_o$ | 0.29±0.03 (3) | 0.3±0.03 (4) | 0.11±0.06 (5) | 0.51±0.19 (2) | 0.21±0.87 (3) |

**Fig. 2.** Single channel analysis of reconstituted ICln in a membrane build of DiphPC, and mixtures of DiphPC and phosphatidylcholines with acyl-chain length of C12 and C18. In panel A, an original tracing is shown of ICln reconstituted in DiphPC/DLPC (1:1) recorded at the voltages indicated. In panel B, an original tracing is shown of ICln reconstituted in DiphPC/DSPC (1:1) recorded at the voltages as indicated. C: The conductance (GpS) and open probability ($P_o$) for ICln channels reconstituted in DiphPC/DLPC (1:1), DiphPC/DMPC (1:1), DiphPC, DiphPC/DPPC (1:1), and DiphPC/DSPC (1:1) bilayers.

conductances are +44.70 ± 3.25 mV (n=10; Diph-PC/DMPC 1:1) and +46.13 ± 5.60 mV (n=18; Diph-PC/DLPC 1:1) as well as 2.30 ± 0.3 pS (n=4) and 2.47 ± 0.48 pS (n=3), respectively. In order to test if a further increase of the amount of DLPC in the membrane could further increase the $E_{rev}$ value and therefore the selectivity for potassium, we used the tip-dip method which allows the current to be resolved to single channel events and furthermore allowed to establish membranes with a higher DLPC content. The maximum ratio of Diph-PC/

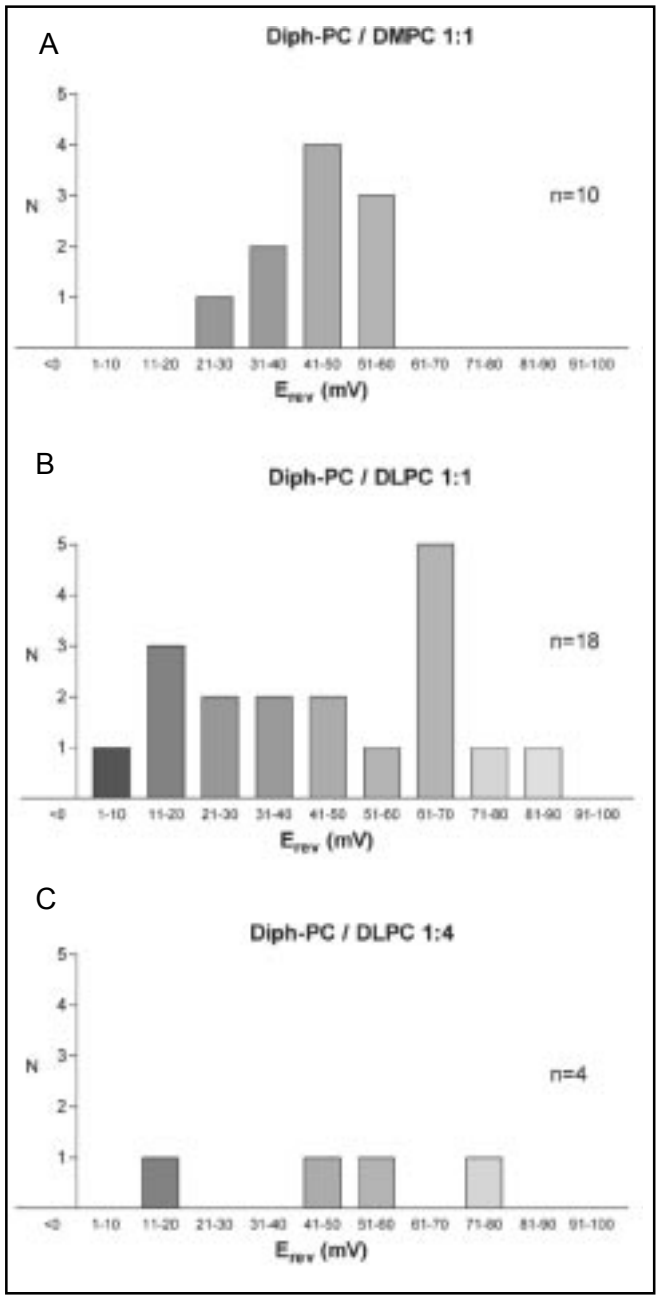



**Fig. 3.** Reconstitution of ICln in mixtures of different lipids. A) Reconstitution of ICln in Diph-PC/DMPC 1:1 B) Reconstitution of ICln in Diph-PC/DLPC 1:1. C) Whereas for the experiments summarized in panels A and B (and also in figure 1) the macro-bilayer setup was used for the experiments summarized in (C) the tip-dip technique (see Material and Methods) was employed. The tip-dip technique enables the increase of the amount of DLPC used for membrane formation, i.e. to a ratio of 1:4.

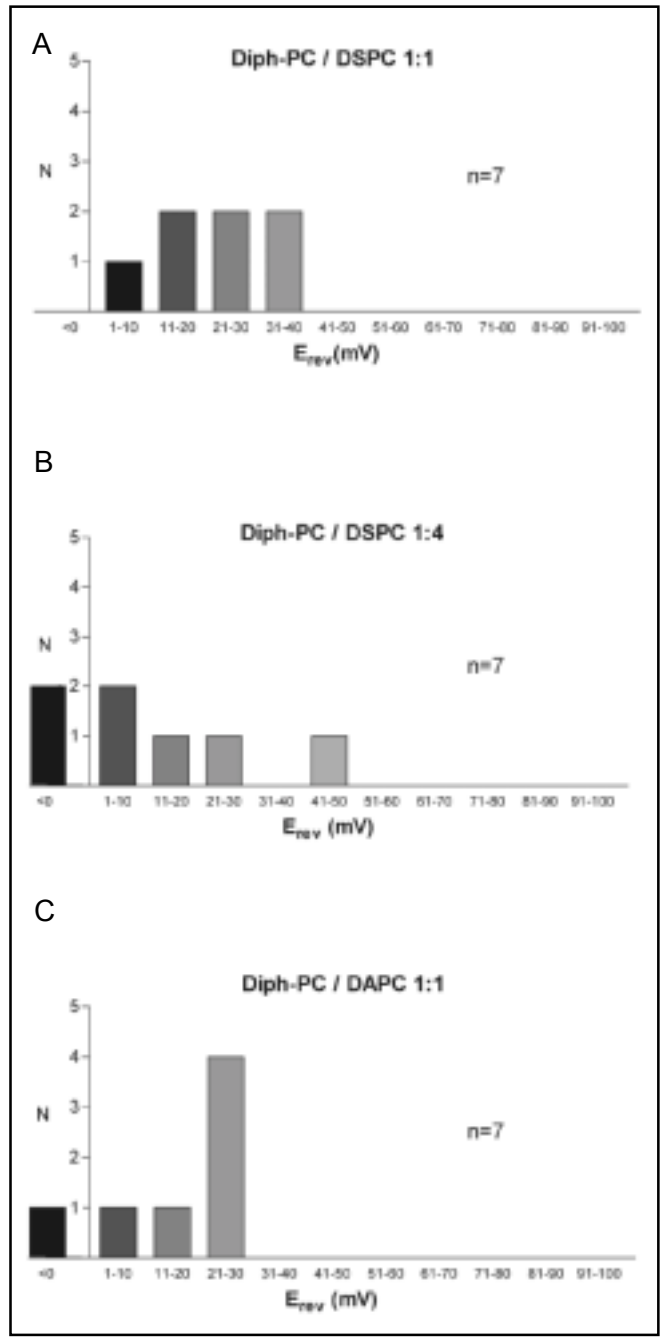



**Fig. 4.** The reconstitution of ICln in bilayers with an acyl-chain length of >16C leads to currents that are more chloride selective if compared to the bilayers build of phosphatidylcholines with an acyl-chain length of <16C. A) Reconstitution of ICln in a 1:1 mixture of Diph-PC/DSPC. B) Increasing the amount of DSPC in the membrane to a ratio of 1:4 further reduces $E_{rev}$, and leads to chloride selective currents in two out of seven experiments. C) Increasing the saturated acyl-chains to 20C did not further reduce $E_{rev}$ if compared to Diph-PC/DSPC (1:4).

DLPC yielding stable membranes, was 1:4. Using this Diph-PC/DLPC ratio the $E_{rev}$ value as well as the single channel conductance, again, did not change significantly (+48.49 ± 12.86 mV; n=4; 1.2 ± 0.3 pS, n=3) if compared to the value obtained if Diph-PC/DLPC (1:1) is used for membrane formation (figure 3).

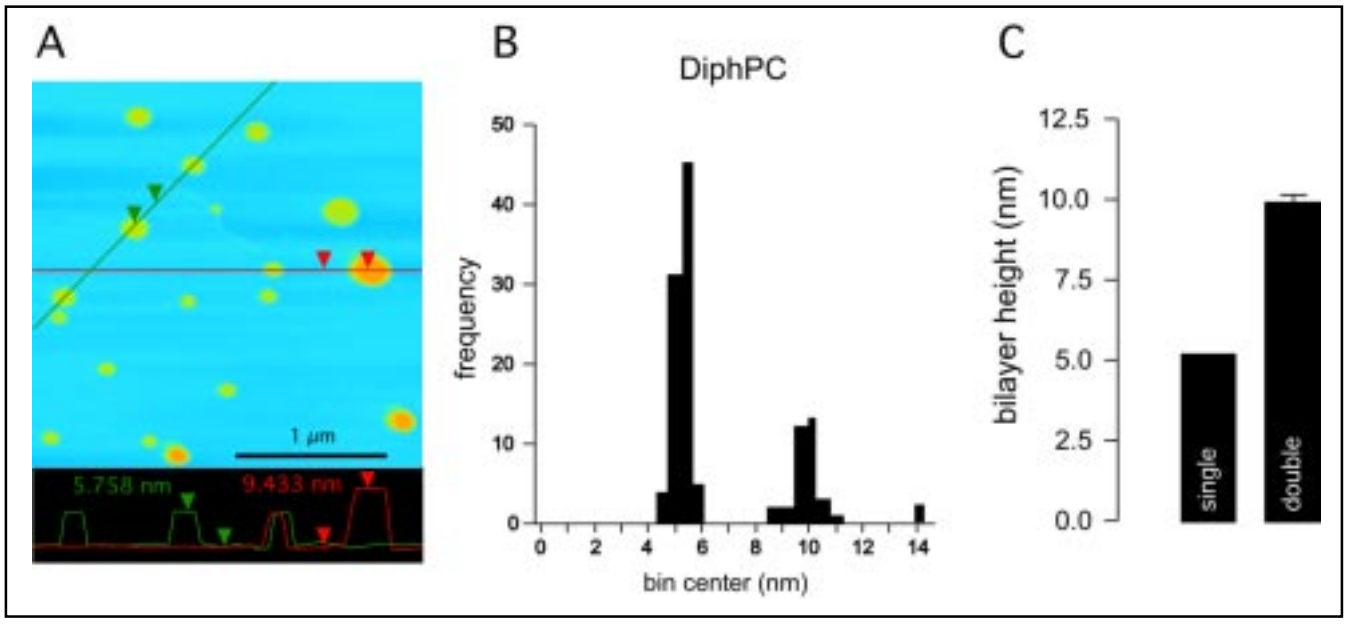



**Fig. 5.** Measurements of the membrane thickness of pure Diph-PC bilayers using the AFM. A: Images of supported Diph-PC bilayers prepared from lipid vesicles as described in the Methods section. Most of the bilayers displayed a circular or elliptical barrel shaped structure. Two distinct heights could be detected as indicated by the pseudocolors: a small-size population (yellow), which represents single Diph-PC bilayers, and a higher-size population (orange), which corresponds to double bilayers. The lower panel shows two height section profiles along the red and green lines shown in the upper panel. The given height differences refer to the locations as indicated by the green and red arrows, respectively. B: Frequency distribution histogram of Diph-PC bilayers heights (n=120). C: Mean heights of Diph-PC bilayer. The left (single, n=85) and right (double, n=35) bars represent the thickness of single and double bilayers and correspond to the two populations of bilayer heights given in panel B.

We also tested the elongation of the acyl-chains from C16:0 (Diph-PC or DPPC) to C18:0 and C20:0. Whereas the shortening of the acyl-chain length from C16:0 to C12:0 leads to an increase of the potassium selectivity of reconstituted ICln channels, as shown above, increasing the acyl-chain length to C18:0 (1,2-distearoyl-sn-glycero-3-phosphocholine; DSPC) or C20:0 (1,2-diarachidoyl-sn-glycero-3-phosphocholine; DAPC) leads to more chloride selective ICln channels.

As shown in figure 4, the use of DSPC (Diph-PC/DSPC 1:1) leads to a significant change of $E_{rev}$ to less positive values (+22.31 ± 4.81 mV; n=7) if compared to Diph-PC/DPPC (1:1). The switch of $E_{rev}$ to less positive values corresponds to a change of the channel selectivity from potassium towards chloride. However, as shown in figure 2, the single channel conductance did not change (2.80 ± 0.6 pS, n=3). The increase of the amount of DSPC in the membrane to Diph-PC/DSPC 1:4 did not significantly reduce $E_{rev}$ (+10.87 ± 11.84 mV; n=7) any further, however, this manoeuvre revealed in two experiments highly selective chloride channels with $E_{rev}$ values of –41.92 mV and –7.88 mV, which correspond to a pK/pCl value of 0.13 and 0.70, respectively. As also shown in figure 4, a further increase of the acyl-chain length from 18C to 20C did not further change $E_{rev}$ (+12.19 ± 6.69 mV; n=7; Diph-PC/DAPC 1:1). As in the case of Diph-PC/DSPC (1:4), also under this condition, in one experiment out of seven, a chloride selective current appeared ($E_{rev}$ = -22.77 mV, corresponding to a pK/pCl of 0.36).

Using the atomic force microscope, the membrane thicknesses of Diph-PC alone and the DiphPC/DAPC lipid mixture in vesicles were measured (figures 5 and 6). As shown in figure 6, the two lipids are not homogeneously mixed, but form ‘rafts’. Preparation of pure DiphPC vesicles led to the formation of lipid bilayers with a mean apparent height of 5,28±0,03 nm (n=85). In addition, a second population with a mean apparent height of 9,99±0,19 nm (n=35) could be identified, most likely representing double DiphPC bilayers (figure 5).

As shown in figure 6, mixtures of DiphPC-DAPC at a molar ratio of 1:1 similarly led to the formation of single and double lipid bilayers with an apparent mean height of the lowest detectable level of 5.62±0.15 nm (n=52). This value is slightly but significantly higher compared to the value obtained from pure DiphPC

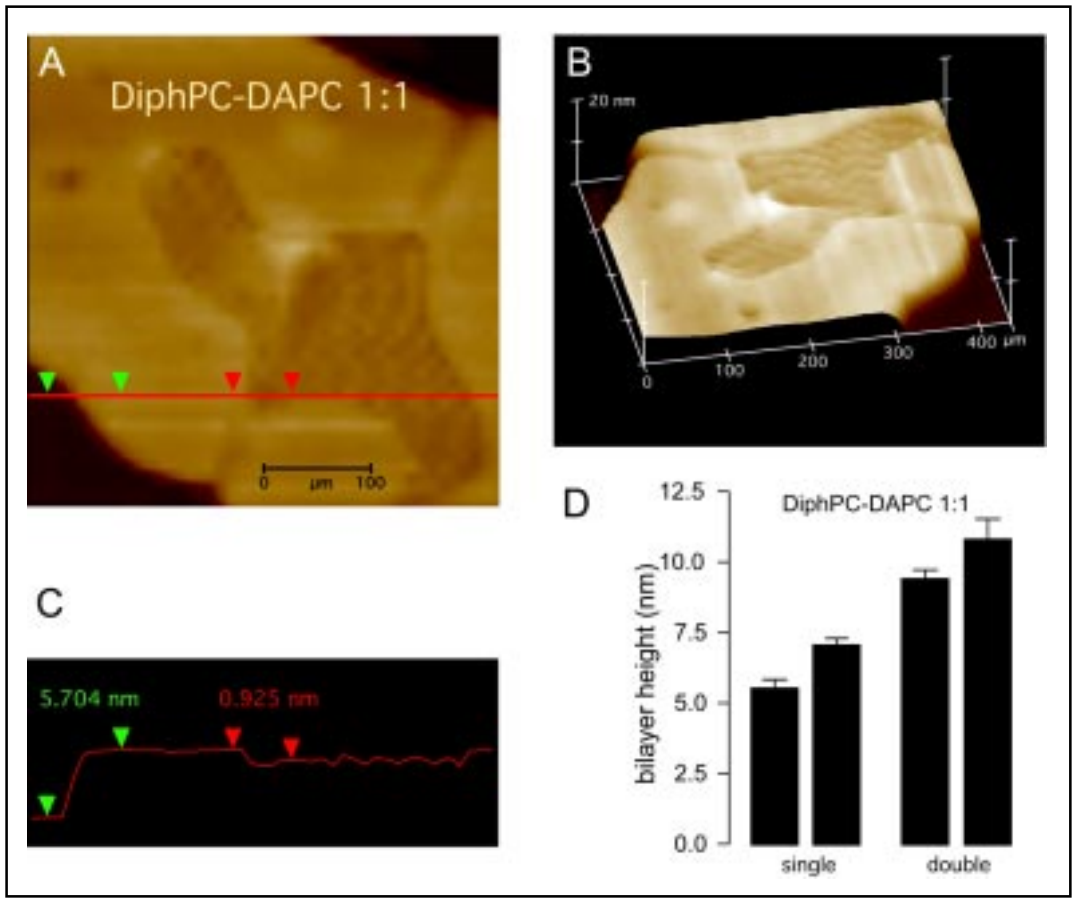



**Fig. 6.** AFM measurements of bilayers composed of a 1:1 molar mixture of Diph-PC and DAPC. A and B: 2D and 3D images of a lipid raft embedded in a basal bilayer with a thickness of ~5.7 nm. The raft is approximately 1 nm thinner than the surrounding bilayer and represents either a Diph-PC bilayer embedded in a surrounding symmetric DAPC or an asymmetric Diph-PC-DAPC bilayer embedded in a pure DAPC bilayer. C: Height section profiles along the red line shown in panel A. The given height differences refer to the locations as indicated by the green and red arrows, respectively. D: Mean heights of bilayers composed of Diph-PC and DAPC. The left two bars (single) and right two bars (double) represent the thicknesses of single and double bilayers, respectively. As evident, two discrete heights can be measured in both single and double bilayers. These height differences arise from lipid rafts as those shown in A-C. The mean height differences of these rafts are similar in single and double bilayers and amount to ~1.6 nm.

bilayers. Approximately 80% of these bilayer formations contained sharp edged islets and/or rims of a discrete higher level with an average value of 7,13±0,15 nm (n=40), the measured mean difference in height averaging 1.65±0.09 nm (n=40). Approximately 50 % of these basal bilayers supported additional bilayers with a mean height of 9.51±0.25 nm (n=26), again containing islets and/or rims with an average height of 10.93±0,57 nm (n=10) and a mean height difference of 1.57±0.22 nm (n=9).

## Discussion

Our current understanding of the plasma membrane evolved from the late nineteenth century, when Overton observed - and experimented with - cell boundaries and speculated on the lipid nature of the permeable barrier-structure, to the early 1970's when Singer&Nicolson proposed the 'fluid mosaic model' of the plasma membrane (for a detailed historical analysis of cell-membrane bilayer research see [21]). The idea that the plasma membrane is not a homogenous lipid layer, but rather an inhomogeneous boundary (in terms of chemical composition) separating the cell interior from the outside world became pertinent [22, 23]. Other features of the membrane lipids, which have been appreciated only lately, are their high lateral mobility [24, 25], their ability to cluster into domains [9, 26, 27], and the dazzling high turnover the membrane lipids can undergo. Steinman et al. [28] showed that the entire plasma membrane of some cells can be renewed within one hour by endo- and exocytosis. The lipid double layer of the cell membrane is not a perfect barrier and therefore some molecules can cross *per se* the 'plain' structure leading to an exchange of solvent and solutes between both sides of the membrane. However, in order to make the exchange efficient, proteins are immersed into the bilayer in order to allow molecules and/or information to pass [1]. It becomes more and more

evident that most of these transmembrane proteins are not located within lipid domains or 'rafts' but are rather located in the boundary regions of the raft-like-domains [22, 29]. These transmembrane proteins, as the lipid molecules in which they are immersed, show a high lateral mobility and can therefore shift from one lipid neighbourhood to another. Since the lipid environment influences the three-dimensional structure of the immersed proteins [9] - it was shown, e.g. that the activity of the KcsA potassium channel depends on the surrounding lipids [10] - it is intriguing to think, that by shifting from one lipid environment to another, ion channels could also shift their selectivity and/or gating property. This scenario also suggests the possibility that stress applied onto the membrane - which in turn is able to modify its biochemical and biophysical properties [30] - could be translated into a response element for ion channel function. Such a hypothesis, as was proposed by Kinnunen [31], is particularly appealing for the regulatory mechanism involved in cell volume sensing and consecutive ion transport activation in order to restore the original volume after shrinkage or swelling of the cells, a process also known as regulatory volume increase (RVI) or regulatory volume decrease (RVD) [32-37]. In order to obtain some insights into the dynamic interaction between channel forming proteins and the surrounding lipid environment we set out to investigate whether or not different acyl-chain lengths of phosphatidylcholines are able to modify the selectivity of the ICln ion channels [18]. These channels are potential candidates for the molecular entity forming the ion permeating structure of the regulatory-volume-decrease-channels (RVDC), a class of ion channels which are still not unambiguously defined on a molecular level [38]. Recently we have shown that the reconstitution of ICln in lipid bilayers built of Diph-PC yields potassium selective ion channels with a single channel conductance of 2.4 pS (pK/pCl = 4.2; [16]). The selectivity of the reconstituted channels was shifted towards chloride (but still remained potassium selective) in the presence of calcium, and the amino acids of ICln, responsible for the calcium binding, have been identified [16]. The reconstitution of the purified ICln channel proteins in a bilayer made of native heart lipids (in the presence of calcium), however, gave rise to chloride selective ion channels [15]. These findings suggested, that the lipid environment in which the channels are embedded could change their selectivity. Here we show, that indeed the length of the acyl-chains of the phosphatidylcholines, used for bilayer formation, are able to shift the selectivity of the reconstituted pores from potassium to chloride, without changing the single channel conductance of reconstituted ICln. Reconstituting ICln in DiphPC ($C16:0[(CH_3)_4]$) leads to potassium selective currents. Omitting the methyl-groups (DPPC; C16:0) or the shortening of the acyl-chains of the phosphatidylcholines to C14:0 (DMPC) and C12:0 (DLPC) leads to an increased potassium selectivity of the reconstituted currents, whereas an increase of the acyl-chain length to C18:0 (DSPC) and C20:0 (DAPC) produced a significant shift towards chloride selective currents, and, in some experiments, revealed currents which are perfectly chloride selective even in the absence of calcium. The mechanism(s) by which this shift in selectivity is provoked is unclear, however, we assume that by increasing the acyl-chain length and therefore the bilayer thickness, the two predicted extracellular loops of the ICln protein, when modelled as a transmembrane channel, would narrow the pore orifice, and allow the two arginines (one arginine per loop amounting to a total of four if a homodimeric structure of the channel forming ICln is considered [18]) to select anions for the permeating ion species. The channel-model assumed [18, 37] for this working hypothesis is still speculative, however, several point mutations leading to well defined changes of the biophysical behaviour of the reconstituted channels [16, 17], as well as NMR studies which revealed the 'molten-globule' structure [39] of the ICln protein [40], sustain the loop-hypothesis of the changed selectivity of ICln reconstituted in different lipid environments. Most of the reconstitution-experiments presented here were produced in the mixtures of two lipids. Therefore, a reason for the appearance of a mixed selectivity phenotype i.e. potassium as well as chloride selective currents, as shown in figures 4 b and c, could reflect the fact that distinct lipids do not form homogeneous lipid layers but rather separate in different domains [9, 26, 27], and therefore, transmembrane proteins could be embedded in the different domains or the respective boundaries. Here we show, using the atomic force microscope (AFM), that the lipids used (DiphPC/DAPC) do indeed form distinctive rafts. AFM has proven to be an excellent technique to study lipid micro-domain (raft) formation [41-44].

As shown in this study, analysis of height profiles of low hydrated pure Diph-PC bilayers by AFM revealed a mean thickness of ~5.3 nm (figure 5). This value is in good agreement with results obtained from various other methods (~5.2 nm) (reviewed in [45]) and AFM measurements, which range from ~5.5 nm [46] to 6.0-7.0 nm [47] of the thickness of bilayers formed by DPPC, which has an identical number of carbon atoms in the

hydrocarbon chain (C16:0). Some bilayers supported a second bilayer. The thickness of these double bilayers were 9.99 ±0.19 nm and therefore somewhat smaller than expected from the double thickness of a single bilayer of 5.28 ± 0.03 nm (figure 5c). This is in contrast to AFM measurements of double DPPC bilayers, where the double bilayer exhibited more then double the height of the basal one [47]. This may be explained by the low hydration state of the bilayers investigated in the present study which accounts for a reduced lamellar repeat spacing of neighbouring bilayers due to reduced interlamellar water space [45]. Using a 1:1 molar mixture of Diph-PC and DAPC a slightly but significantly higher value was obtained for the lowest detectable height level compared to the thickness of pure basal Diph-PC bilayers, suggesting that the basal bilayers of the mixtures were formed either by pure Diph-PC bilayers, pure DAPC bilayers or asymmetric Diph-PC/DAPC bilayers, thus giving rise to the slightly increased mean thickness of the basal bilayer (figure 6d). Similarly to pure Diph-PC bilayer the formation of double bilayers occurred in the lipid mixtures. Clear domain (raft) formation could be observed in both basal and top bilayers in most of the preparations investigated. These domains are formed by sharp edged islets and/or rims within and/or surrounding a given bilayer with a mean height difference of ~1.6 nm. An example for such a raft is shown in figure 6. Given the difference of 4 carbon atoms in the acyl chains of Diph-PC and DAPC (C16:0-C20:0) the expected height difference for rafts in fully hydrated bilayers is approximately 0.7 nm [2]. The higher measured mean value of ~1.6 nm in this study may be explained by the low hydration state of the samples which causes thickening of the bilayer [2] and is in good agreement with AFM height measurements for raft formations in lipid mixtures of DMPC-DSPC (C14:0-C18:0; 1.3 nm) [48], DLPC-DSPC (C12:0-C18:0; 1.8 nm) [49], DMPC-DSPC (C14:0-C18:0); 1.6 nm [47] and DPPC-DLPC (C16:0-C12:0); 1.4 nm) [46].

In conclusion, our experiments demonstrate that the selectivity of reconstituted ICln depends on the lipids used for bilayer formation. The shorter the acyl-chains and therefore the thinner the double layer of the membrane, the more potassium selective the channels are. In contrast, the longer the acyl-chains are and therefore the thicker the membrane, the more chloride selective the channels become. Although these findings might be a peculiarity of the ICln channels, these experiments point towards the possibility that the membrane, besides offering a special-determinant, could also be involved in mechanisms modulating channel function, a regulatory mechanism pertinent whenever the biophysical and/or biochemical characteristics of the plasma membrane are changed.

## Acknowledgements

We thank J.M. Edwardson, N.A. Geisse for helpful discussion and A. Wimmer for technical assistance. This work was supported in part by the Austrian Science Foundation (P14102-med), Austrian National Bank and by the Italian Science Foundation.

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