RECONSTITUTION OF ION CHANNELS

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I. INTRODUCTION

The first model of the structure of cell membranes was developed very early from indirect evidence.[1] It postulated the existence of a bimolecular lipid membrane which serves as the main and electrical isolating part of the cell membrane. The proteins were either associated with or incorporated into the membrane.[2] Following this idea, it was postulated that the proteins being incorporated into the bimolecular lipid matrix (integral membrane proteins) were the source of ion transport across the electrical isolating lipid membrane.[3] Ion channels formed by these proteins were one of the mechanisms being considered to be involved in the ion transport across cell membranes.

These ion channel-forming proteins were proposed to be involved in the electrical excitation of biological membranes very early.[4] For a long time, however, it was impossible to investigate directly the properties of these channels, i.e., in single channel experiments. Limited by the low current and time resolution of electrophysiological measurements, only multichannel experiments were possible. Due to the fact that multichannel data can be explained by a larger number of single channel models there was a need for detailed single channel data to obtain a more in-depth view of channel function. In addition, all of the single channel parameters calculated from the electrophysiological experiments were scattered over a wide range of values.

Nevertheless, from electrophysiological experiments a large number of different ion channels were postulated to be active in biological membranes, and highly specific properties were given to these hypothetical ion channels. However, until 1962 not even the existence of single ion channels could be shown experimentally.

Following that line of thought, the first technique for the formation of planar lipid bilayers was established in 1962 by Mueller et al.,[5] using large amounts of organic solvents to stabilize the artificial membrane.

The first single channel fluctuations shown were those of EIM and gramicidin in planar lipid bilayers which were formed according to that technique.[6-9] For a period of time, single channel fluctuations being induced by different polypeptide antibiotics and related substances[10] in planar bilayers were the only really measured single channel fluctuations.

At that time it was not possible to measure current fluctuations of single ion channels in cell membranes directly. It was obvious that the incorporation of channel-forming proteins into planar lipid bilayers would be a useful technique for finding the proof of the existence of single ion channels in biological membranes and for investigating the properties of these hypothetical channels in single- and multichannel experiments. Most of the early reconstitution experiments were not very successful. Problems occurred because these bilayers contained large amounts of organic solvents, techniques had to be developed to incorporate

proteins, which were not water soluble, into planar lipid bilayers, and there were no suitable methods to extract ion channel-forming proteins from biological membranes.

In 1972 Montal and Mueller succeeded in forming planar bilayers containing only small amounts of organic solvent.[11] They folded two monolayers from the air-water interface of aqueous solutions to a bilayer on a solvent pretreated hole. The monolayers were spread from highly volatile solvent lipid solutions on the air-water interfaces. Before folding the bilayers, most of the solvent was allowed to evaporate. This new technique partially solved the problem of the solvent contents of planar lipid bilayers according to Mueller et al.[5]

Montal and co-workers then tried to incorporate ion channel-forming proteins (e.g., partially purified rhodopsin-lipid complexes) into bilayers formed by this method. By adding the protein lipid complexes to the lipid phase they found ion channel activity.[12]

Somewhat later, a technique to incorporate channel proteins into planar bilayers was developed by Miller and Racker,[13] using the fusion of protein-containing vesicles with planar lipid bilayers. Through this technique, for example, channel proteins from the sarcoplasmic reticulum[14,15] were incorporated successfully into solvent-containing planar bilayers according to Mueller et al.[5]

Schindler developed another technique to form virtually solvent-free planar lipid bilayers.[16] He used monolayers, which formed spontaneously on the air-water interface of lipid vesicle suspensions, to form bilayers on pretreated holes in Teflon® septa. This technique offered as well an easy way to incorporate proteins into the planar bilayer by using protein containing lipid vesicles to form the monolayers. For example, the matrix protein from *Escherichia coli* outer membranes was found to form a voltage-gated channel in planar bilayers by Schindler and co-workers.[17]

A variety of other ion channel forming proteins from different membrane preparations were reconstituted thereafter using the various methods mentioned above (for details see Section III).

In 1981 Boheim et al. succeeded in forming stable bilayers from pure PC-lipids at below the phase transition temperature, i.e., in the frozen state.[18] The incorporated channel proteins into these bilayers in the functional state,[19,20] by the fusion of protein-containing vesicles with these bilayers which were formed according to Montal and Mueller. This development, together with the success of the fusion of vesicles with PE-doped PC-bilayers, formed according to Montal and Mueller,[21] opened other new techniques to reconstitute channel-forming proteins into planar bilayers.

All of the first reconstituted channel proteins originated from membrane fragment vesicles or from only partially purified proteins.

By the incorporation of ion channel-forming proteins into planar lipid bilayer arises the possibility, however, to incorporate highly purified proteins into membranes of a well-defined lipid composition. Using only one protein and one pure lipid, this gives the chance to investigate a two-component system, a possibility not given in biological membranes with their unknown highly complicated composition. Only for very simple systems, however, is there a possibility for a complete theoretical description.

To take advantage of this, it was necessary to develop suitable methods to purify channel-forming membrane proteins from biological sources and to incorporate them into lipid vesicles. This was first done with the acetylcholine receptor protein[22,23] on a scale which gave some meaning to bilayer experiments. Nelson et al.[24] and Boheim et al.[19] carried out experiments reconstituting the purified acetylcholine receptor protein into planar bilayers in the functional state; i.e., they found ion channels being formed by this protein, which were very similar to the ion channels known from other experiments. Until now, only a few other highly purified ion channel forming proteins have been reconstituted successfully, with the TTX binding protein forming the electrophysiological well-described sodium channel[25-28] being one of these.

Comparison of data from reconstituted purified channel proteins with data from the same channels being reconstituted from membrane fragments and with electrophysiological results has given a deeper understanding of channel function.

Parallel to these developments a new technique was found to investigate single channels directly in biological membranes (the patch-clamp technique).[29-31] Through this a number of new aspects was introduced into the investigation of membrane transport processes. As channels can be investigated directly in the biological membranes from which they originate, it is no longer necessary to reconstitute them into planar bilayers in order to obtain single channel data. Not all membranes, however, are easily accessible for this technique, and problems can arise because of the highly complicated composition of biological membranes (see above). Due to this there still exists a number of problems in the investigation of ion channel properties, which can only be solved by the different reconstitution techniques.

The technical evaluation accompanying the patch-clamp technique initiated new developments in bilayer and reconstitution experiments. Tank et al.[32] patched directly, for example, large protein-containing lipid vesicles, thus combining some advantages of both techniques, i.e., the high resolution of the patch-clamp technique and the possibility of using membranes of well-known lipid composition as is the case for the reconstitution techniques. They thus avoided the problem of incorporating proteins from vesicles into the planar bilayers, as it is rather easy to incorporate lipid-soluble proteins into the membranes of lipid vesicles.

Besides that, techniques were developed to form bilayers on the tip of patch-clamp pipettes[33-37] introducing other new aspects in reconstitution work.

Mainly by using single channel data obtained from bilayer reconstitution and from patch-clamp experiments it was possible to obtain more detailed theories of channel gating.[21,38-42] The further development of these theories using data from physically and chemically well-defined systems (i.e., from planar bilayers) will be one of the major tasks of future reconstitution work.

II. METHODS OF PLANAR LIPID BILAYER FORMATION AND PROTEIN INCORPORATION INTO PLANAR LIPID BILAYERS

In order to carry out successful bilayer reconstitution experiments a few technical preparations are necessary. Therefore, before considering the properties of single reconstituted ion channels, we will discuss in this section some aspects of the equipment which nearly all planar bilayer reconstitution experiments have in common.

A. Membrane Support Devices

A drawing of a generalized bilayer setup is given in Figure 1. It shows all of the necessary components of both the mechanical and electronic equipment. Most of these components are commercially available. Special attention must be given, however, to some of them.

1. Divided Chambers

For all bilayer experiments, a divided chamber is necessary. The two parts of the chamber (usually made from Teflon®) are electrically isolated from each other except for one small hole on which the bilayer is formed. The most simple method is to drill a small hole (diameter mm) into the wall separating the two parts of the measuring chamber. This simple technique works adequately, however, only with bilayers containing larger amounts of solvent. The solvent here may be necessary to compensate the unevenness of the drilled hole.

2. Teflon® Septa

A more advanced technique is that of working with two separate chambers. Both have a small hole in one side, through which they are connected. Between both chambers a special

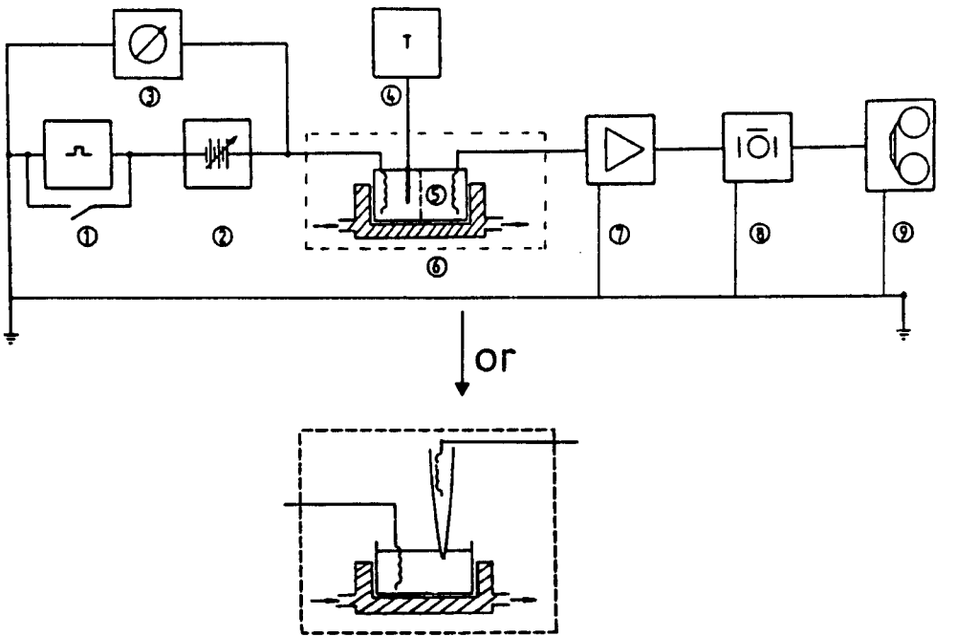

FIGURE 1. Block diagram showing the principles of a bilayer measuring set-up. (1) Function generator; (2) source for variable voltage; (3) voltmeter; (4) temperature measuring system; (5) membrane, Teflon® chamber, aqueous solution, and electrodes; (6) thermostat; (7) current-voltage converter with high current resolution; (8) oscilloscope; and (9) recording system (FM tape recorder, pen recorder, etc.). The same set up can be used for experiments with bilayers on the tip of patch pipettes or for patch-clamp experiments when the membrane is changed to a pipette system.[5]

Teflon® septum is pressed in, with the bilayer carrying hole being part of it. The characteristics of the hole determines mainly the rate of success in bilayer experiments, so special attention must be given to its formation. Earlier researchers used thin Teflon® foil and burned holes into it. Later, Schindler developed a more advanced technique.[43] The septum was melted together from one very thin (6 μm) Teflon® foil and two rings of thicker Teflon® material. The thicker material can be glass-fiber coated for even better electrical and mechanical stability. This also yields a smaller electrical capacitance in the experiments. Into the inner thin foil the membrane carrying hole can be melted by use of a heated platinium needle, cut by a special tool, made by electric sparks or by other various methods. Using these techniques it is possible to make holes for bilayer formation from about 50 μm to 1 mm in diameter. They have been used mainly together with the Montal technique[11] to form bilayers.

3. Glass Pipettes

As pointed out above, the development of the patch-clamp technique influenced the technique of bilayer formation. Methods were found to form bilayers on the tip of glass pipettes. The quality of the tip of the glass pipette determines mainly of course the quality of the bilayer being formed on it. The pipettes are prepared in the same manner as in patch-clamp experiments. Procedures on how to pull and firepolish these pipettes have been described in the literature in detail.[44,45]

4. Electronic Equipment

As can be seen in Figure 1 the majority of the electronic equipment used in bilayer

experiments is commercially available standard equipment. Due to the small currents which have to be measured in single channel experiments (down to less than 0.1 pA), a very high quality current measuring circuit is necessary. Earlier these current amplifiers were usually built by the researchers themselves from operational amplifiers with very high input impedance (more than 10^{12}). After the patch-clamp technique became well known in all electrophysiological laboratories, state-of-the-art current amplifiers became commercially available.[46] They have a current resolution of 0.1 pA together with a time resolution of about 1 msec. Time resolution can be chosen as high as 50 μs when a high current resolution is not necessary.

Data from an experiment, i.e., current fluctuations of channels or other data, are usually stored on an FM tape recorder for further evaluation with a computer using adequate A to D converters.

B. Methods of Bilayer Formation

As already noted in the introduction there have been several different methods of bilayer formation developed. All of these methods have been the starting point for ion channel reconstitution experiments. The ones of greater importance will be described in the following section in more detail.

1. Bilayers Formed on Teflon® Partitions

As pointed out above, bilayers are usually formed on holes in Teflon® partitions between the two parts of the measuring chamber. These holes can be drilled directly into Teflon® walls or made more sophisticated in Teflon® septa. Presently, holes drilled in Teflon® walls directly are used mainly for solvent containing bilayers.

a. Lipid-Decane Bilayers

The oldest method to form planar bilayers is the one introduced by Mueller et al.[5,47] The lipid is dissolved in a nonvolatile organic solvent. In most experiments decane is used for this purpose. A drop of the lipid solution is brought mechanically onto a lipid pretreated hole. For this reason usually thicker Teflon® walls are used to drill the holes into. The drop is spread mechanically to a very thin film, which shows rainbow colors in reflected light. After waiting a certain amount of time, part of the solvent (decane) diffuses out of the film and, spontaneously, black spots are to be seen on the film in reflected light. When the entire film is so thin that it looks black in reflected light the planar bilayer has formed (black films). The current view of these bilayers is that they still contain a good deal of organic solvent which may be distributed randomly in solvent lenses and that only part of them are really bimolecular lipid films. Figure 2a presents a diagram of this method.

Depending on the amount of solvent present in these bilayers, they are on the average thicker than pure bimolecular films, thus yielding a lower capacity in electrical measurements. Bilayers from about 0.1 up to 2 mm diameter can be formed by this method.

b. Lipid-Hexane Bilayers

In 1972 Montal and Mueller developed another technique to form planar lipid bilayers.[11,48] They dissolved the lipid in a highly volatile organic solvent (hexane or pentane) and spread small amounts of this solution on the air-water interface of aqueous solutions. After the solvent had evaporated, the remaining lipid films were moved from below the membrane carrying hole to above it. Thereby, a bilayer was formed by the folding of the preformed two monolayers. Figure 2b shows this technique. As these bilayers contain only very small amounts of organic solvent (virtually solvent-free planar lipid bilayers), they can only be formed on well-prepared holes in Teflon® septa as described in Section II.A.2.. Nevertheless, even these holes must be pretreated with organic solvent. Usually, solvents like hexadecane

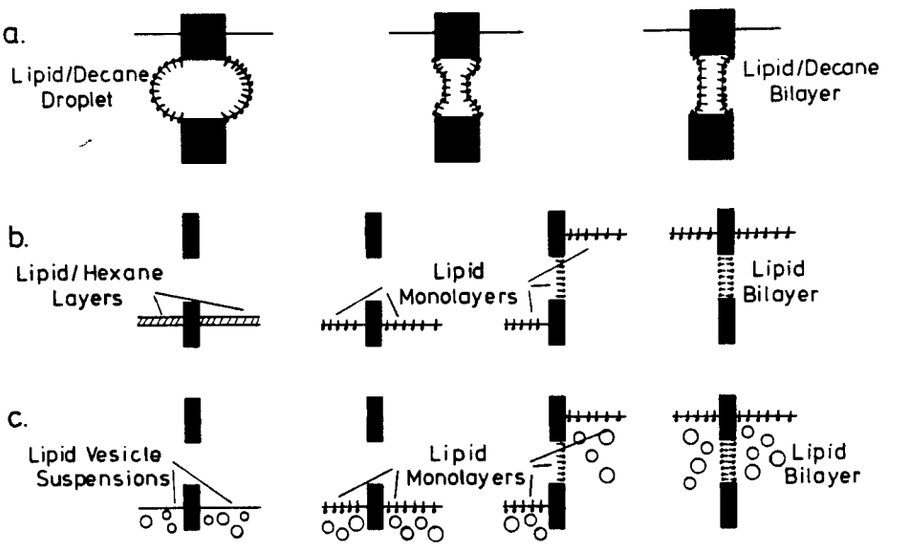

FIGURE 2. Diagram of formation of bilayers: (a) is the Mueller technique for the preparation of painted black bilayers from lipid decane solutions; (b) is the Montal technique for the preparation of virtually solvent-free planar bilayers from solutions of lipid in highly volatile organic solvent; and (c) is the technique introduced by Schindler to prepare virtually solvent-free planar lipid bilayers by using the formation of lipid monolayers on the air-water interface of vesicle suspensions. The monolayers on the air-water interface are taken as being in equilibrium with the membranes of the vesicles in the subphase.

or squalene are used which are soluble in lipid in only very small amounts. They form a torus which stabilizes the bilayer-to-wall connection. As the bilayers do contain only small amounts or organic solvent, their capacity is close to that of pure bimolecular lipid films. They can be formed on holes from about 50 to 500 μm in diameter.

c. Bilayers from Lipid Vesicles

Here lipid monolayers are formed spontaneously on the air-water interface of lipid vesicle suspensions.[49,50] These monolayers are in equilibrium with the vesicles in the aqueous solution.[51] Schindler[16] made use of this fact in another method of bilayer formation. Here vesicle suspensions are situated on both sides of the membrane carrying hole at the start of a bilayer experiment. After monolayers have formed spontaneously and are in equilibrium with the subphase, they are moved from below the hole to above it. A bilayer is thus formed as shown in Figure 2c by the folding of two preformed monolayers very similar to the technique of Montal and Mueller. The Teflon® septa carrying the holes must be pretreated in the same way as described for Montal bilayers. An advantage of this technique is that the bilayers so formed contain even less solvent than those formed according to Montal and Mueller. However, there are still small amounts of solvent present in these bilayers which originate from the pretreatment of the hole.

2. Bilayers Formed on the Tips of Glass Pipettes

After the development of the patch-clamp technique, it was evident that this technique had some advantages over the bilayer techniques in addition to the fact of working with natural membranes. The membrane areas on the tip of a glass pipette are in the range of approximately 1 μm^2. This small area enables a much better signal-to-noise ratio in the measurements[52] as compared to that of standard bilayer techniques. In addition, no treatment of the pipette tip is necessary, so that the membranes on it are not affected by organic solvents from this source.

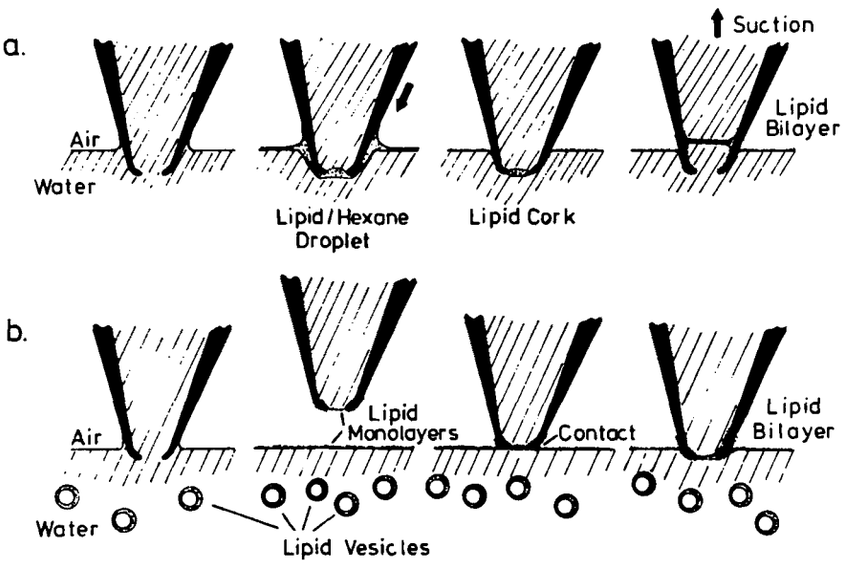

FIGURE 3. Part (a) illustrates the technique of forming a bilayer on the tip of a patch pipette starting with a drop of lipid hexane solution on the tip of the pipette. First, a drop is applied to the pipette and the pipette is dipped into the aqueous solution. Then it is sealed by the drop of lipid solution. Applying suction to the pipette, the drop is thinned until it spontaneously forms a bilayer. Part (b) illustrates the forming of Montal-type bilayers on the tip of a patch pipette by starting with a monolayer spread from a lipid hexan solution. The bilayer itself is formed by moving the tip of the pipette repeatedly through the monolayer. This should be done very gently, the best results being obtained when using a micromanipulator. The same technique is used when the monolayer is formed by self-assembly from a vesicle suspension.

In order to combine the advantages of standard bilayer techniques, which includes free choice of membrane composition and the construction of systems containing only one ion channel forming protein species, with that of the patch-clamp technique with its high time and current resolution and possibility of working with absolutely solvent-free membranes, it was necessary to work out techniques to form lipid bilayers on the tip of glass pipettes. This has been accomplished by a number of groups.[32-37] We will comment on the different approaches used in the following section.

a. Solvent-Containing Bilayers

The simplest way to form lipid bilayers on the tip of a glass pipette is to apply a small drop of lipid solution (lipid dissolved in hexane) to the tip.[33] To form a bimolecular bilayer from this drop, the tip of the pipette is moved into the bath solution and then suction is applied to it by using a special pipette holder. At the beginning a drop of lipid having a small capacity due to its thickness seals the pipette. After applying suction, this drop becomes thinner and a bilayer is formed. The process can be monitored by capacity measurement or even by visual inspection under an appropriate microscope. In Figure 3 a diagram of the technique is given. This method of bilayer formation gives, in addition, the possibility of changing the area of a bilayer within one experiment in a controlled manner, which is not possible with any of the other techniques. The area of the bilayer can be measured by measuring its capacity.

Due to the fact that this method starts with a drop of lipid solved in hexane, the bilayers formed by it may still contain some organic solvent. A pretreatment of the pipette, however is not necessary. Standard firepolished pipettes, as used for patch-clamp experiments, are sufficient and yield seal resistances far in the Gohm range.

Another possibility is to form the bilayers on the tip of glass pipettes from monolayers on the air-water interface of aqueous solutions.[33-35] These monolayers or multilayers can be spread from lipid hexane solutions as described for forming planar lipid bilayers with the Montal technique. When spreading the lipid, the tip of the pipette is submerged deeply under the surface. After the solvent has evaporated the pipette is gently moved repetitively through the surface monolayer. For optimal results a micromanipulator is used. From this a bilayer is formed on the tip of the pipette. Figure 3b shows a rough sketch of this technique. These bilayers may contain some solvent due to the fact that the solvent may not evaporate quantitatively. Otherwise, they can be treated as described above.

b. Solvent-Free Bilayers from Lipid Vesicles

As mentioned above, forming planar bilayers on tip of glass pipettes gives one the possibility of working absolutely solvent free, as there is no need for pretreating the tip of the pipette.

For this purpose, the monolayer on the air-water interface of aqueous solutions is formed by self-assembly from a vesicle suspension, as described in part 1.c of this section. The bilayer on the tip of the pipettes can then be formed as described in the previous section.

The monolayer in equilibrium with the subphase is formed spontaneously on the air-water interface of a lipid vesicle suspension. During this time the pipette tip is situated outside of the solution. After the formation of the monolayer the pipette tip is gently moved through the surface repetitively.[34,36] This can be done most effectively by using a micromanipulator. Usually the bilayers are less stable at the beginning, but become more stable in the course of time. Otherwise, these bilayers can be treated as described above, and as shown in Figure 3b.

Schindler developed an advanced form of this technique.[37] He also used monolayers on the air-water interface of lipid vesicle suspensions for the formation of the bilayers, but the contact of the two monolayers forming a bilayer was guided by a mechanical setup. This method leads to more stable bilayers than that described above and gives the possibility of controlling separately the internal pressure of the two monolayers from which the bilayer is formed. A disadvantage of the technique described by Schindler are the extensive technical preparations necessary to support this type of bilayer formation.

c. Patched Large Lipid Vesicles

The patch-clamp technique makes use of the fact that a limited area of a cell membrane can be electrically isolated at the tip of a glass pipette. Techniques have been developed to even patch small single cells in suspensions.[55,56] Mono- and multilamellar lipid vesicles can be made in sizes of up to several micrometers in diameter.[57,58] This has been the reason for trying to apply the patch-clamp technique directly to large lipid vesicles.[32] The same equipment as in patch-clamp work can be used and the methods of patching the vesicles are identical to those of patching isolated cells. For convenience the lipid vesicles can be immobilized before they are patched using various methods.

C. Reconstitution Techniques

A large number of techniques has been developed to incorporate ion channel forming proteins and polypeptides into planar lipid bilayers. Each of these techniques can be used together with bilayers formed according to several of the techniques of bilayer formation as described above. In the following section we will describe some of the aspects for the reconstitution of ion channel proteins from biological membranes into planar lipid bilayers. We will indicate the type of bilayer that is most efficient with the particular method being mentioned.

1. Water-Soluble Proteins

Proteins which are partially water- and lipid-soluble are very easy to incorporate into planar lipid bilayers. The bilayer is formed according to any of the techniques described above. Then the protein can be added to the aqueous phase in its water-soluble form. It is incorporated into the bilayer by diffusion to the bilayer and from the water phase into the lipid phase. This process can be enhanced, among other possibilities, by the application of an appropriate potential across the planar bilayer.

2. Fusion of Protein-Containing Vesicles with Planar Bilayers

Most of the ion channel proteins deriving from biological membranes are not water soluble. They cannot be directly added to the water phase.

Usually, however, these proteins can be incorporated into the membranes of lipid vesicles. Other ion channel forming proteins cannot even be isolated from their native membrane. Usually they are present in the membranes of membrane fragment vesicles after the biochemical procedure. A way has to be found to incorporate these proteins into planar bilayers.

One method to transfer the proteins from vesicle membranes to planar bilayer membranes is the fusion of the protein-containing vesicles with planar bilayers. Different methods can be used to realize such fusion experiments. In all cases the bilayer is preformed by one of the methods described above.

The vesicles are added to the aqueous phase on one side of the bilayer. This side is usually indicated as cis side. The fusion of the vesicles themselves with the bilayer is aided through the use of various technical tricks, as the rate of spontaneous fusion of vesicles with planar lipid bilayers without any additional treatment is very small.

a. Through the Use of PS Lipids and Millimolar Amounts of Ca^{++} in the Aqueous Phase

Miller[13] developed a technique to fuse vesicles with solvent-containing planar lipid bilayers according to Mueller and Rudin. The bilayers he used were composed of PE-lipids, PC-lipids, and large amounts of negatively charged lipids (as, for example, PS-lipids). In the aqueous phase around the bilayer, millimolar amounts of Ca^{++} were present. In addition the cis side was hyperosmotic to the trans side or the vesicles were hyperosmotic according to the bath solution. All of the given conditions were necessary to obtain measurable fusion rates and the fusion rate itself depended on the amount of Ca^{++} present in the aqueous phase and on the grade of hyperosmolarity. In Figure 4a a diagram of this technique is shown. Figure 4b shows a current recording from one of these experiments. After addition of Ca^{++} to the bath solution under conditions otherwise identical to those given in the figure legend, the vesicles from the SR started to fuse with the planar bilayer. Each fusion comes along with the incorporation of one or more ion channel forming proteins. Thus, the conductance of the bilayer to the permeable ion is increased stepwise by each fusion. At a constant membrane potential this is measured as a stepwise increase in the current flowing across the bilayer. This is seen clearly in the figure.

b. Through the Use of PC Lipids Below Tc

In 1980 Boheim et al. developed techniques to form reproducibly planar bilayers from pure PC lipids below the phase transition temperature of these lipids.[18] It was known that vesicles tend to fuse spontaneously at a measurable rate at the phase transition temperature of the lipid used.[59,60] The same principle was used to fuse vesicles with planar bilayers.[19,20] The following results were obtained from these experiments. A hyperosmolarity of the cis side or the vesicles is also a prerequisite for fusion under these conditions. The bilayers must be composed from pure lipids below their phase transition temperature. Until now only synthetic mixed-chain PC lipids have been tested to form bilayers below Tc in a greater number of experiments, so that no data are available concerning other lipids. Especially

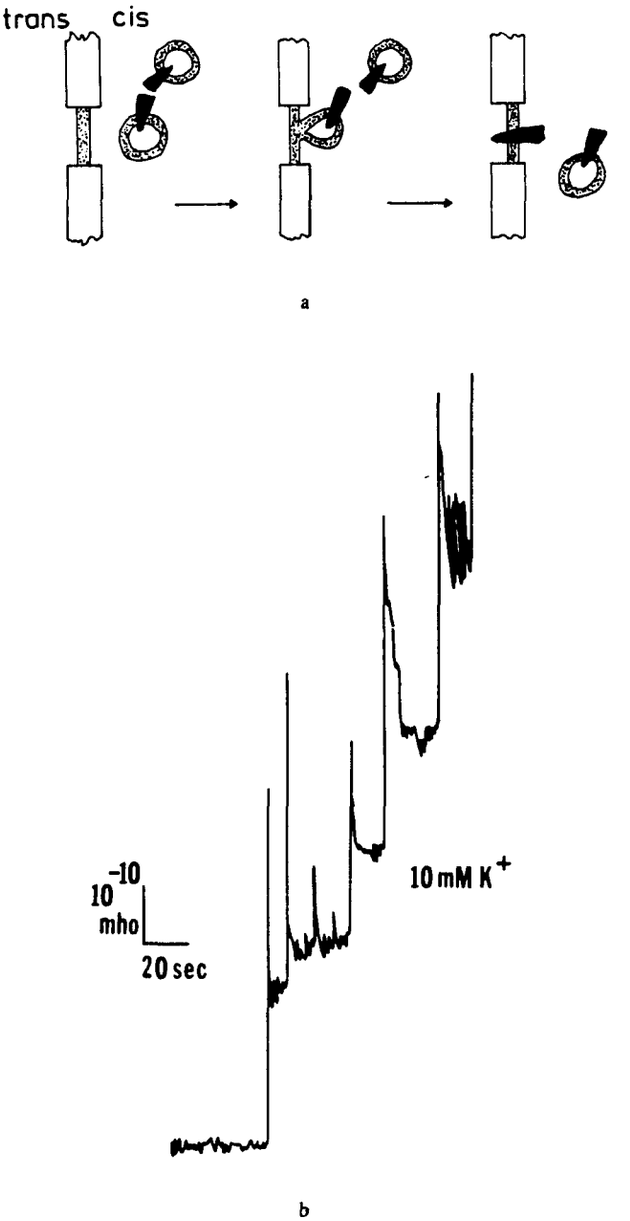

FIGURE 4. Vesicles may be fused with a planar lipid bilayer containing negatively charged lipids as shown schematically in part (a) of this figure. As calcium is a prerequisite for this technique, the fusion only occurs when a certain amount of calcium is present on the vesicle-addition side. Part (b) shows the fusion of vesicles from the sarcoplasmic reticulum starting after the addition of vesicles to a bath solution containing 0.7 m*M* calcium. Simultaneously, the current across the bilayer is monitored at a fixed potential (25 mV). The current increases step-wise after vesicle addition to the cis side. Each fusion event can be identified by the current transient at its occurrence. The aqueous solution contained 10 m*M* potassium in this experiment. (From Miller, C., *J. Membr. Biol.*, 40, 1, 1978. With permission.)

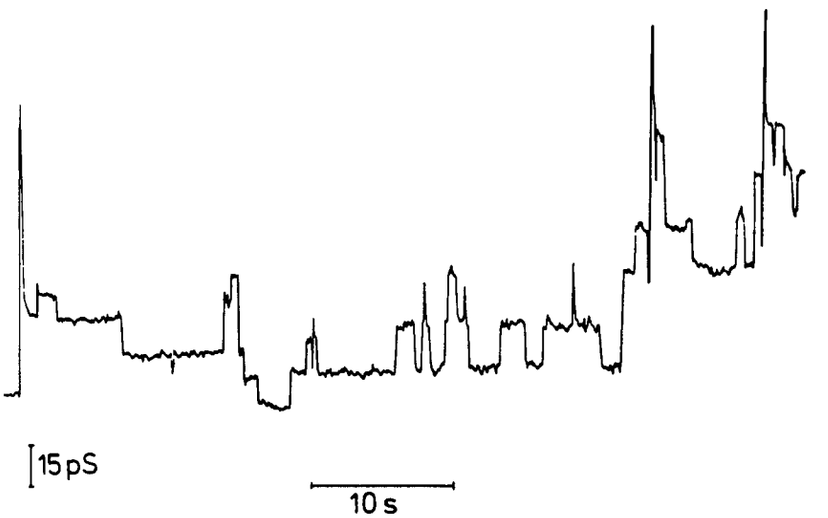

FIGURE 5. Current fluctuations of cationic channels from cilia from *Paramecium tetraurelia*. The three current transients indicate three single fusion events of vesicles with a bilayer made from pure 1,3-SMPC at 22°C, that is below the phase transition temperature of the pure lipid of 30°C. By each fusion some channels are incorporated into the planar bilayer as can be seen by the current fluctuations after the fusion events. The aqueous solution did not contain any calcium to induce the fusion.

1,3-SMPC (1-stearoyl, 3-myristoyl, glycero, 2-phosphatidylcholine), but other lipids, too, were found to give good results in fusion experiments. Due to the fact that stable bilayers can be formed below the lipid phase transition temperature only according to the method of Montal, no experiments have been possible with decane-containing bilayers. Figure 5 shows a recording from a fusion experiment using vesicles of membrane fragments from paramecia cilia. At the conditions given in the legend to Figure 3, subsequent fusion events are shown by an increase in the transmembrane current at a fixed potential. By each of the fusion events some ion channel proteins are incorporated into the planar bilayer, the single channel fluctuations after each fusion event are clearly resolved. In addition to this, each fusion comes along with a current transient. Thus, fusion events can be clearly identified (see Figure 4 also).

c. Through the Use of PE Lipid Doped PC Bilayers

From the techniques described above the question arose as which conditions were sufficient for successful fusion experiments. Experiments were done, using Montal type virtually solvent-free planar lipid bilayers of lipid compositions as used in Miller's fusion experiments. It was found that negatively charged lipids and Ca^{++} in the aqueous phase were not necessary for fusion under these conditions. With decane-containing planar bilayers the same also holds. In order to get measurable fusion rates it was sufficient to use high amounts of PE lipids in PC lipid bilayers. An osmotic gradient was still a prerequisite for fusion. While in bilayers using PC lipids in the fluid state (i.e., above phase transition temperature), high amounts of PE lipid were necessary (60 to 80%), the fusion rate using PC lipid below its phase transition temperature was already perceptibly higher using only small amounts of PE lipid (Hanke and Boheim[141]). This fact is especially important when bilayers containing cholesterol are used, which is necessary for certain proteins.[19,22] Even small amounts of cholesterol present in the planar bilayer decrease the rate of fusion dramatically. As a result of this, conditions had to be found to increase the fusion rates in the presence of cholesterol.

A conclusion from the above described experiments was that the fusion rates using solvent containing bilayers according to the technique of Mueller and Rudin were sufficiently higher than with virtually solvent-free planar bilayers under any conditions. Although decane thus seems to increase the fusion rates, its use is limited as the effects of higher amounts of organic solvents on the properties of the ion channels which are to be reconstituted must be investigated in any specific case.

3. Assembly of Protein-Lipid Monolayers from Vesicles on an Air-Water Interface

The technique of bilayer formation described in Section II.B.1.c. opens another way of incorporating proteins into planar bilayers. The bilayer is formed from two monolayers which have formed spontaneously on the air-water interface of aqueous solutions. The composition of these monolayers should resemble somewhat that of the vesicle membranes in the subphase.[22] Schindler used this idea to incorporate proteins into planar lipid bilayers.[16] In his experiments vesicles already containing the proteins in their membranes were used to form the monolayers from which the bilayers were made. Thus at the moment of the formation of the bilayer, the proteins were already present in it. In more sophisticated experiments the internal pressure of the monolayers can be controlled in addition.

4. Incorporation of Nonwater-Soluble Proteins into Bilayers on the Tips of Glass Pipettes

All of the techniques mentioned above can also be used with bilayers on the tips of glass pipettes.[33-37] It must be taken into account, however, that fusion rates and incorporation by diffusion (Section II.C.1.) are also dependent upon the area of the bilayers used. The very small areas of bilayers on the tips of glass pipettes therefore limit the use of these techniques.

Figure 6 shows two principle ways in which proteins can be incorporated into bilayers on tips of glass pipettes. As the rates of incorporation after addition of vesicles to either the bath solution or the inner side of the pipette were remarkably high, other mechanisms in addition to the ones described above may also play a role in the incorporation of proteins in bilayers on the tips of glass pipettes.

5. Patched Large Protein-Containing Lipid Vesicles

An elegant way to investigate the properties of ion channel proteins in bilayers is to patch large protein-containing lipid vesicles.[32] As proteins usually are easy to incorporate into the membranes of lipid vesicles this is a suitable way to investigate reconstituted ion channel proteins. Tank et al.[32] demonstrated that this approach does work. In order to obtain larger vesicles, which are easier to handle, they applied a freeze-thaw cycle to their vesicle preparation before use. This trick yields vesicles up to 30 μm in diameter which are easily accessible for standard patch-clamp techniques. The large multilamellar vesicles one obtains are usually immobilized for convenience before patching them.

III. RECONSTITUTED ION CHANNEL PROTEINS

The study of membrane transport functions with electrophysiological methods always ends up with macroscopic data about the involved processes. Very often the data are best explained by postulating an ion channel of specific properties to be the pathway for the ions through the hydrophobic core of the bimolecular lipid matrix. However, from these macroscopic data single channel parameters can only be calculated by applying certain preformed models of the ion channel to the evaluation of the data. Of course, the results obtained for the parameters of the postulated single channels depend strongly on the model used. In addition, not only the data are to be investigated, but also data about other processes which being present, because of the complicated composition of biological membranes are obtained. The researcher then is in the situation of having to isolate the wanted data by other procedures.

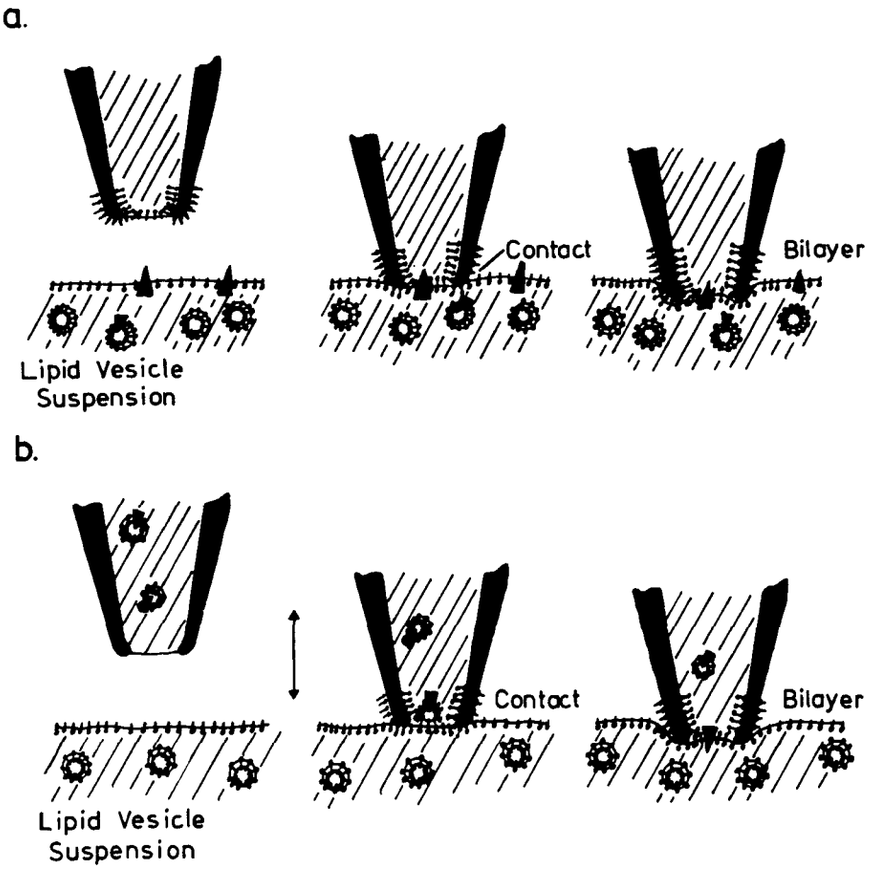

FIGURE 6. Two possibilities are shown schematically of how to incorporate ion channel-forming proteins into bilayers on the tip of patch pipettes. The protein can be incorporated via a monolayer on the air-water interface as shown in (a) or by fusion of vesicles with the bilayer. The vesicles can be added to the inside of the pipette as shown in (b) or to the bath solution (not shown here). When the vesicles are added to the interior of the pipette they will sink down to the bilayer, especially when they are loaded with some sucrose, for example, thus giving a higher probability for fusion. In addition to the fusion process, vesicles may be incorporated into the lipid film (bilayer) coating the pipette after a certain time. They may diffuse laterally into the bilayer on the tip of the pipette. This effect may give a much larger area for fusion and thus better fusion rates than would be given for the bilayer on the tip of the pipette itself.

One way often used in this case is the pharmacological determination and blockade of certain ion channels.

The patch-clamp technique ends up very often at a certain point with the same dilemma caused by the complicated nature of the investigated object, the cell membrane.

The only way to solve this problem until now is through the biochemical isolation of a certain membrane protein and the investigation of its specific properties independent of the composition of the cell membrane. But, by having a sample of isolated protein the problem is not solved, as there is still the question of how to study the transport properties of an isolated protein. The reincorporation of the protein into a membrane of a well-defined and known composition seems to be a suitable way to do that.

For example, the protein can be incorporated into lipid vesicles and these can be investigated in flux measurements. This has been done extensively with some proteins.[61-64]

To obtain a more in-depth view of the transport properties of the protein investigated, the protein can be incorporated into a planar lipid bilayer of defined lipid (and protein) composition. Here the transport properties of the protein can be investigated in detail in a defined environment. Data can be obtained for ion channel forming proteins, for example, in single- and multichannel experiments. Furthermore, in comparison with the data from the native membrane, questions can be answered about the effects of the biochemical procedure on the specific properties of the protein investigated.

Before proceeding in the manner described above, the resesarcher may attempt to investigate the properties of a certain protein in reconstitution experiments without it first undergoing a biochemical purification procedure to be sure about the functioning of his reconstitution essay.

In addition to this there are a large number of interesting ion channel proteins which yet have not been isolated biochemically.

Therefore, very often proteins are reconstituted from membrane fragment vesicles without further purification. Although a part of the native membrane is reconstituted together with the protein which is to be investigated, a good deal of information can be obtained from such experiments (in any case, the membrane composition is better specified than that of the majority of the native membranes).

One should always keep in mind that a lot of membranes in biological systems are not accessible to any of the electrophysiological techniques, including the patch-clamp technique. If the researcher wishes to study hypothetical ion channels present in such membranes (for example, the membranes of discs in the outer segments of visual cells), the only way to do it, is to reconstitute them into artificial membranes.

For the reasons given above, we will clearly discriminate in the following sections between reconstitution of highly purified proteins and reconstitution of less-pure proteins.

1. Highly Purified Proteins

Until now, only a few ion channel forming proteins have been investigated in reconstitution experiments in the highly purified form.

The acetylcholine receptor protein can be purified because of its pharmacological property of binding to α – bungarotoxin.[19,23] This has been done and the protein has been reconstituted by various researchers.[19,23] Thus it has been shown that the reconstituted purified protein shows about the same properties as was expected from other experiments.[19] With the acetylcholine-receptor protein we have the advantage that data are available from electrophysiological and pharmacological experiments as well as from biochemical studies. In addition extensive single channel data are available about the ion channel formed by this protein from patch-clamp experiments. This gives necessary information about the function of this protein in its natural environment. This unique situation makes the protein a favorite candidate for reconstitution experiments. By experiments with it the effects of biochemical and reconstitution essays may be studied.

Another protein purified as a result of its pharmacological properties is the sodium channel protein taken from a variety of exitable membranes.[26-28,65,66] Planar bilayer reconstitution experiments using this purified protein have been recently published.[67-69] They contained some single channel data not expected from the well-known electrophysiological data (see Section III.B. for details), as well as first results comparable to electrophysiological data.[68]

Other than those just mentioned, only a few additional proteins from various sources have been used in reconstitution experiments in the highly purified form, purified proteolipids from different sources being one group of them. We will briefly comment on some of these proteins in the following section.

2. Channel Proteins from Membrane Fragment Vesicles

There are a number of reasons for reconstituting ion channel proteins from membrane

fragment vesicles into planar bilayers, as was pointed out already in detail. The most simple one is that in the earlier stages of reconstitution experiments no preparations of purified proteins were available. Therefore, all reconstitution techniques were developed by using membrane fragment vesicles. This lack of preparations with a high degree of purification still holds true for the majority of the investigated ion channel forming proteins. Another reason is to obtain data from these experiments for comparison with those derived from experiments with highly purified proteins. Last not least, membrane fragment vesicle preparations containing a certain ion channel forming protein are usually much easier to obtain than highly purified preparations.

A large number of different ion channel proteins have been reconstituted by using membrane fragment vesicle preparations as starting material. For the majority of these ion channels, some more detailed data are therefore available now concerning not only the behavior of the single channel but also about multichannel systems. Not very much is known, however, about the biochemistry and molecular properties of these proteins. Usually, nothing is known about the structure of the protein and very often there are even doubts about the physiological relevance of the investigated channels or at least about some of their properties.

B. Classification of Reconstituted Channels

In this section we are going to describe the specific properties of ion channels as obtained from reconstitution experiments.

By each of the proteins we will briefly comment on the different techniques of bilayer formation and protein reconstitution used with the particular protein being discussed. In addition we will, whenever possible, compare the results obtained with data from other methods, especially from the patch-clamp technique.

Due to the large number of proteins reconstituted into planar bilayers we are going to focus on those of more general interest. This selection of course may, to a certain extent, reflect the personal interests of the author.

The channels are classified in respect to their electrophysiological and pharmacological properties, especially to the dependence of the channel gating on various experimental parameters.

1. Nongated Channels

Nongated channels are channels which are not significantly dependent on any experimental parameter in their properties, especially concerning their kinetics. This may be a special property of the ion channel-forming protein investigated or simply a consequence from the lack of complete experimental data.

The best described nongated channel is the channel formed by gramicidin in planar lipid bilayers.[6,7] We will not focus on this special channel in more detail, as it is built by a very special pore-forming polypeptide and not involved in very interesting biological processes. Nevertheless, a great deal was learned about the general aspects of porus ion transport by studying the gramicidin channel.

Figure 7 shows current fluctuations of cationic channels reconstituted from membrane fragment vesicles from cilia of *Paramecium tetraurelia*.[70] The experimental conditions are given in the figure legend. This protein was reconstituted into planar bilayers made from PC lipids below the phase transition by fusion of protein-containing membrane fragment vesicles with the planar bilayers. In addition, experiments were carried out with bilayers containing PE and PC lipids.[141] All the bilayers used were made according to the method of Montal and Mueller. The properties of the channel were not significantly dependent upon the voltage applied and only slightly dependent upon the lipid composition of the bilayers used in the experiments.[141] In 0.1 *M* KCl the channel has a conductance of 20 pS and a lifetime in the 100-msec range. The channel is permeable only to cations, but we found that

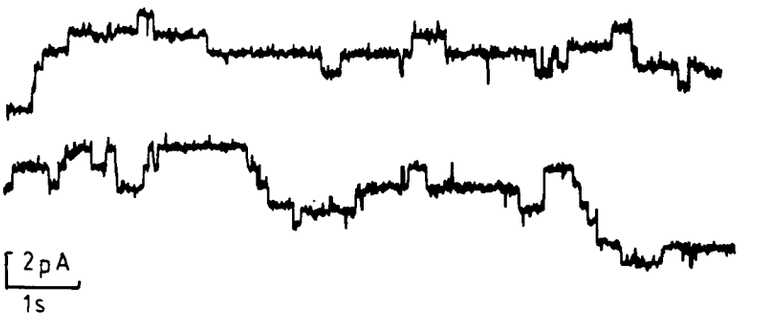

FIGURE 7. Current fluctuations of the cationic channel from cilia fragment vesicles from *Paramecium tetraurelia*. Two traces of single channel fluctuations are shown after fusing the vesicles with a 1,3-SMPC at 22°C. The bath solution contained 100 m*M* potassium. The channel did not show any pronounced dependence on the experimental parameters voltage and presence of divalent cations. The applied voltage was 100 mV for the traces depicted.

it is permeable to divalent cations. This fact opens the possibility that the channel is involved in the calcium transport across the cilium membrane.[70] Outside of this, however, there are no physiological data available dealing with this channel so that ideas concerning its physiological relevance are rare.

An interesting property of this channel, however, is that the channel kinetics are affected by mutations of the paramecium. In pawn mutants[71] of the paramecium, a channel comparable to the presented one is still present, but its kinetics are much faster.[70,71]

Another example of a channel which is (at the present point of knowledge) nongated, is a sodium-selective channel from bovine rod outer segment membranes.[72] The above-mentioned channel is slightly voltage dependent at very high potentials. This may be true for the cationic channel from paramecia, too, but nothing is known about the nature of this voltage dependence and its physiological relevance.

2. Voltage-Gated Channels

In this section we will discuss channels in which the kinetics are voltage dependent, but which are not known to depend on the presence of certain ions or drugs in the bath solution. Of course, the kinetics of all known ion channels depend on the ionic strength of the bath solution, but this effect will not be discussed here (this holds for the above section, too).

One group of channel proteins forming voltage-dependent ion channels is that of the porins. For example, the matrix protein from membranes of *E. coli* was found to form such channels by Schindler and Rosenbusch.[17] They used the method of bilayer formation from protein-containing monolayers on the air-water interface of vesicle suspensions (Sections II.B.1.c and II.C.3). After the formation of the bilayer, channel fluctuations could be initiated by application of an appropriate high voltage. The initiation appeared to be irreversible. After the initiation period the channels did not disappear, but were found to be highly voltage dependent. When high positive voltages were applied, the channel closed. In a series of experiments after initiation the applied voltage was set to zero and then jumps to different voltages were applied. After each voltage jump from zero to the final potential, the time course of the inactivation of the channels was recorded. The higher the voltage jump, the faster the inactivation. Schindler calculated a steady-state current voltage curve from such experiments. The steady-state current voltage curve showed an initial part of positive resistance which was ohmic at the beginning. This part reflects the ohmic behavior of the single channel unit conductance.[17] Single channel fluctuations have been clearly resolved in the traces shown in the experiments presented, so that the single channel parameters could

be determined. At high voltage the steady-state voltage curve shows partly negative resistance due to the closing of the single channels. The probability of this channel being in an open state Po is thus clearly demonstrated to be voltage dependent. The channel had a single channel conductance of $\lambda = 0.14$ nS in 0.1 NaCl. It was shown that the smallest conductance that could be induced consisted of three unit steps.[17]

Benz et al.[73,74] incorporated the matrix protein in its water-soluble form (with detergent) using solvent containing membranes (Sections II.B.1.a and II.C.1). They could resolve the incorporation of single ion channels into the planar bilayer, too. Whereas the single channel conductance was found to be about the same in their experiments as compared to those of Schindler, they did not find a closing of the channels at high potentials.

A potassium-selective channel of high single channel conductance ($\lambda = 140$ pS in 0.1 m*M* KCl) has been found by the fusion of membrane fragment vesicles with preformed PS lipid containing decane bilayers. The vesicles originated from SR-membranes.[14,15] Miller and co-workers[14,15] found that a smaller number of resolvable channels were incorporated into the bilayer after every fusion event. Figure 8a shows such ion channel current fluctuations after the incorporation of protein by fusion. Again, the probability of this channel to be in the open state was found to be voltage dependent. The voltage dependence of the channel gating is reflected by the conductance voltage curve of a multichannel system given in Figure 8b, taking into account again that the single channel conductance was not found to be voltage dependent.

From the single channel data as well as from multichannel experiments done with planar bilayers, a detailed model of the channel has been developed. The pharmacological properties of this channel have been studied in detail by Miller and co-workers.[75,76]

To neither of the voltage-dependent channels described above can a clear physiological function be associated. No single channel experiments have been described for the channels discussed above except bilayer reconstitution. A totally different situation holds for the so-called sodium channel.

The sodium channel of excitable membranes was one of the first channels postulated to be active in excitable membranes. Therefore it has been studied by electrophysiological experiments very early.[25-28] Very specific properties were attached to this channel from these older experiments. One of them was the activation-inactivation cycle that the channel was postulated to undergo after an activating voltage jump.

The sodium channel became one of the most extensively studied voltage-gated channels. Data for comparison with results from reconstitution are now available from biochemical, electrophysiological, flux, and patch-clamp measurements. The following results were found: the channel is closed at all constant potential conditions and, therefore it is observable only after activating voltage jumps, after which it undergoes the postulated activation-inactivation cycle. A number of different toxins affect the sodium channel in highly specific manners. Through these properties it could be easily identified and a biochemical procedure for its purification has been developed.[65,66]

It resisted, however, any attempt to be reconstituted into planar lipid bilayers for a long time. Krueger et al.[77,78] succeeded recently in reconstituting the sodium channel into solvent-containing planar lipid bilayers by fusion of membrane fragment vesicles with the bilayer (Sections II.B.1.a and II.C.2). The problem with investigating this channel was that it is closed at constant potential conditions all the time. Upon an activating voltage jump, it activates and inactivates within some milliseconds. So the channel can be investigated only by applying voltage jumps to the membrane in which it is incorporated. Due to the fast activation and inactivation this is very difficult in planar lipid bilayers. Krueger et al.[77,78] therefore made use of the fact that the inactivation can be suppressed by the presence of certain neurotoxins in the aqueous solution around the membrane. They used batrachotoxin (BTX) for this purpose. After addition of BTX to the aqueous solution around membranes

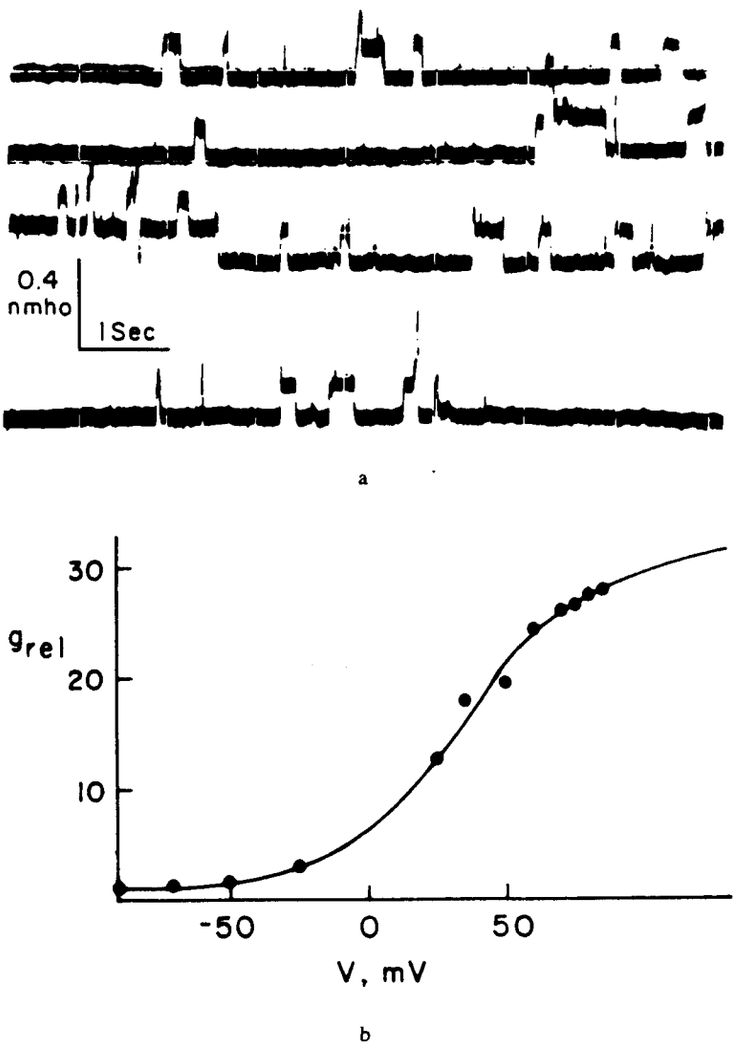

FIGURE 8. Part (a) shows current recordings of potassium channels from the SR after incorporation of the ion channels by fusion of membrane fragment vesicles with a painted planar bilayer as described above. The traces were taken after the fusion had been stopped by taking away the calcium from a bilayer which had only a small number of channels incorporated. The applied voltage was −50 mV and the aqueous solution contained 50 m*M* K_2SO_4. (From Miller, C., *J. Membr. Biol.*, 40, 1, 1978. With permission.) Part (b) shows a current voltage relation of a multichannel system from the SR potassium channel. The fusion of vesicles with the bilayer had been stopped before taking the curve presented and thus the curve represents the steady-state conditions. As the single channel conductance is known to be independent of the voltage applied, the nonlinearity of the presented function must originate from the voltage dependence of the channel gating. Therefore it is clear that the channel closes at high negative potentials. (From Miller, C. and Rosenberg, R. L., *J. Gen. Physiol.*, 74, 457, 1979. With copyright permission of The Rockefeller University Press.)

containing sodium channel protein molecules, they found a continuously bursting channel which was sodium selective and had a unit conductance of $\lambda = 20$ pS in 500 m*M* NaCl.

The terminus burst is used here to describe a specific gating behavior which nearly all known ion channels have in common. The opening events of these channels are not inde-

pendent of each other, but are qued up to so-called bursts (see also Sections III.C.1 and II.E). During the burst the channel has a certain probability (Po) of being in the open state; outside the burst the channel is closed. The burst itself is switched by an additional gating process which is much slower than the gating inside the burst by definition. A probability that the burst is active can be attached to this slow process.

In addition, Krueger et al.[77,78] and Moczydlowski et al.[145,146] investigated some pharmacological properties of this channel in depth and compared them to those which are well known from electrophysiological experiments for the sodium channel. They especially studied the blocking effects of TTX and STX on the reconstituted channel and found approximately the dependence expected from the electrophysiological experiments.

The bursting sodium-selective channel they found is voltage dependent in its inner burst parameters. The dependence of the burst parameters on the applied potential was not shown in their papers. The electrophysiologically described activation-inactivation cycle could not be described by Krueger et al.,[77,78] and Moczydlowski et al.[145,146] because they used BTX to suppress just this property of the channel.

Single channel experiments were done by different groups[79-83] to investigate this channel with the patch-clamp method applied to the membranes of cultured cells. They investigated directly the gating behavior of the sodium channel by applying activating voltage jumps to the membrane. They found single channels in which the conductance and selectivity were comparable to those of the reconstituted channels. In addition, however, the activation-inactivation cycle of the channel was investigated in depth, with and without toxins being present. A lot of data about the channel have been obtained from these patch-clamp experiments; e.g., about its kinetics and pharmacology.[79-83] All data from reconstitution experiments must be compared with these data and it seems to be necessary to ask for good agreement before speaking of successful reconstitution.

Hanke et al. recently reconstituted the purified sodium channel protein into virtually solvent-free planar lipid bilayers made from PE-PC mixtures by fusion of protein loaded liposomes with the bilayer (Sections II.B.1.b and II.C.2.c). The channel protein was purified according to its pharmacological and toxin-binding properties.

Their experiments with the purified protein yielded some unexpected results.[67] Two different channel populations were found in a preparation expected to contain only one specific protein from the purification procedure and the toxin-binding properties of the protein being purified.

In Figure 9 current fluctuations are presented of both channel types. One channel type is active within bursts at constant potential conditions without any of the toxins suppressing the inactivation present in the aqueous solution around the bilayers. The selectivity and the unit conductance of this channel type are similar to those shown by Krueger et al.[77,78] and in patch-clamp experiments. In addition, these values resemble the values which were expected from electrophysiological data. The bursting behavior of this channel is very similar to that of the channel described by Krueger et al., but without any toxin present at the membrane. The channel, however, is blocked by specific toxins, such as TTX and STX, and it is affected in its gating behavior by toxins, which are known to be very specific affectors of the sodium channel.

The second channel type found in these experiments, was a channel which shows a specific activation-inactivation sequence after an activating voltage jump and is not active at constant potential conditions, when no toxins are present at the membrane. In this respect, it resembles the physiological sodium channel. However, its conductance is much larger and its selectivity is less than that of the physiological described sodium channel (μ = 150 psec, in 150 m*M* NaCl, selectivity: 2:1, Na^+ to K^+, conductance ratio). The channel was affected by toxins known to affect the sodium channel in very specific ways. STX blocked the channel. At

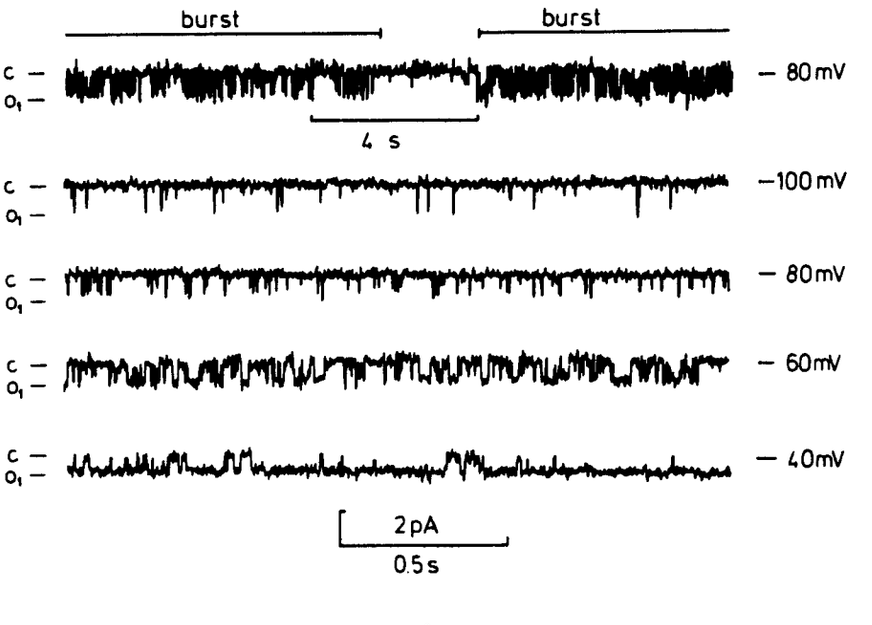

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FIGURE 9. Through incorporation of purified STX binding protein from rat brain synaptosomes into virtually solvent-free planar lipid bilayers made from PE/PC mixtures, two different ion channels are produced in these membranes. Part (a) shows a sodium-selective ion channel of about 25 pS is 150 m*M* NaCl, which is similar to the channel shown by Krueger et al.,[77] except that it does not show any inactivation without toxins being present. The probability of the channel being in the open state within the clearly defined bursts is strongly voltage dependent. Part (b) shows the other ion channel which was found after reconstitution of the same protein preparation. It is a slightly sodium-selective activating-inactivating channel. As the channel is active for only short periods after activating voltage jumps, a series of voltage jumps was applied to the membrane and the current was recorded. After the majority of the voltage jumps short periods of channel activity are found, the length of which are variable. Sometimes no fluctuations are activated by a proper voltage jump. At voltage jumps with the reverse polarity, no channel fluctuations were activated. The unit conductance of the single channel under the same conditions as given for the above sodium-selective channel is $\lambda = 150$ pS. As shown in Reference 67 the two channels depicted in this figure originate from the same protein and may be different states of the same protein aggregate. Transitions from the larger inactivating channel to the smaller sodium-selective channel have been shown in Reference 67. (From Hanke, W., Boheim, G., Barhanin, J., Pauron, D., and Lazdunski, M., *EMBO J.*, 3, 509, 1984. With permission.)

high concentration a scorpion toxin known to slow down the inactivation of the sodium channel supressed the inactivation of this large channel type.[67]

Very recently other groups successfully reconstituted the purified sodium channel protein, too. Hartshorne et al.[69] used a preparation from rat brain. They reconstituted it in a manner similar to the experiments of Krueger et al.[77,78] and obtained comparable results. Again the channel was inactive at constant potential and therefore BTX was used to open it. So their results differ somewhat from those of Hanke et al. The preparation they used contained two small subunits in the protein which were not present in the preparation Hanke et al. used. Besides the differences in the technique of reconstitution, this may be one of the reasons for the differences of the results. Although the comparison of the results of Krueger et al. and Hartshorne et al. gives a remarkable agreement, there are questions about the effect of the organic solvent on the protein as well as questions about the action of BTX and the gating of the channel without BTX being present.

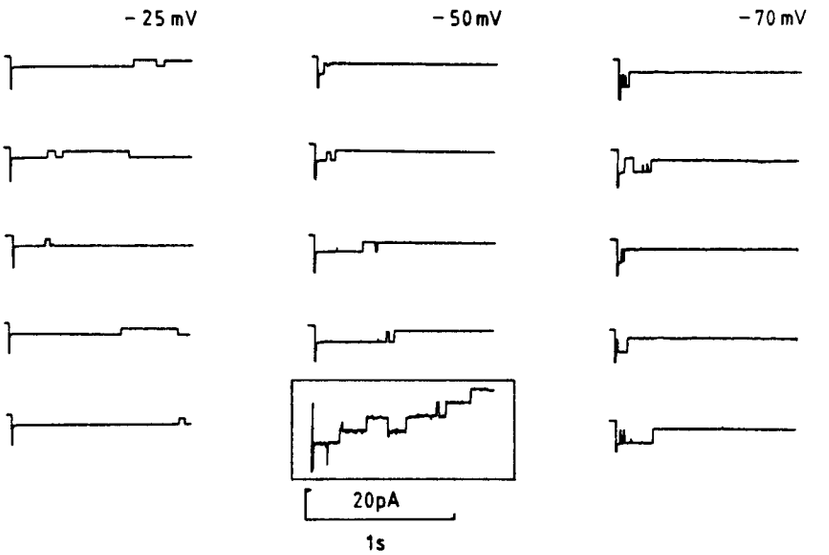


FIGURE 9b.

Of the results of Hanke et al., questions must be asked about the reason for the loss of inactivation and the large conductances of the channels. An explanation for these problems may be that the channels aggregate upon purification or reconstitution. It was found that all conductance steps in the experiments can be explained as multiples of a subunit. Thus the channel would change part of its parameters upon aggregation leading to cooperative gating, loss of inactivation, and perhaps changes in selectivity (Hanke et al.[143]).

Rosenberg et al.[68] recently reconstituted purified sodium channel protein from electroplax of *Electropherus electricus* into the membranes of large liposomes and patched these liposomes. They found single channel activity very similar to that of biological membranes; i.e. they found inactivating channels with fast kinetics without any toxin being present in the aqueous solution. In addition they directly demonstrated the action of BTX on the kinetics of these channels. Their unexpected results were that BTX changed channel kinetics and channel conductance. A possible explanation would be that BTX just enforces an aggregation of the channel proteins thus giving results somewhat comparable to those of Hanke et al.

Although much progress has been made in the reconstitution of the purified and native sodium channel protein into planar lipid bilayers, a lot of questions are still open about these experiments. They only can be answered in further experiments.

However, besides that, a good deal of information about the purified sodium channel protein is available from vesicle reconstitution and flux measurements.[84-86] Mainly data about the binding of drugs to the channel protein and about its biochemistry have been obtained from these experiments, but also some data about the channel gating.

According to the properties of the sodium channel it is not only gated by the voltage, but can also be gated by certain drugs. For example, BTX can open the channel at constant potential conditions, where the channel otherwise is closed. We have discussed the channel in this section and not in the section describing the voltage- and drug-gated channels because in its native membrane it is mainly gated by voltage changes.

Another very important group of voltage-gated channels are the calcium channels from different biological membranes. A lot of these channels show a pronounced activation-inactivation cycle after proper voltage jumps. A good deal of information about the calcium channels has been obtained from patch-clamp experiments.[87-91] Furthermore, we know a lot

about the channels, especially about their pharmacology from electrophysiology.[92-94] The reconstitution of calcium channels, however, still has to fight against principal problems. So it is difficult or even impossible to do experiments with calcium channels in cell-free patch-clamp experiments.[89,90] As is easy to see, the problems in reconstitution are much more difficult then. Nevertheless, recently two papers have been published dealing with the reconstitution of calcium channels from different sources into planar lipid bilayers. Nelson et al.[95] show the reconstitution of calcium channels from rat brain into planar lipid bilayers. They find long lasting (up to s) small single channel events (10 pS in 250 m*M* Ca^{++}). Surprisingly these channels do not run down as would be expected from cell-free patch-clamp experiments. Due to the high calcium concentration the authors used, they could not verify their results by pharmacological tests. So a lot of questions are still open here. Nevertheless, the channels show some properties typical for calcium channels. They are permeable for barium and strontium and can be blocked by micromolar amounts of lanthanum.

Ehrlich et al.[96] showed the reconstitution of calcium channels from cilia membranes. However, the data they present only demonstrate that it is possible to measure single calcium channel fluctuations after fusion of cilia membrane vesicles with the planar bilayer. They found two different types of calcium channels in their experiments with about 5- and 30-pS conductances in 50 m*M* divalent cations under asymmetric salt conditions. Hanke et al.[142] found channels of about 12 and 50 psec in sym. 50 m*M* calcium using a comparable preparation and Montal type bilayers in the frozen state. In contrast to Nelson et al.[95] both groups worked with low concentrations of divalent cations. So their work demonstrates that it should be possible in future experiments to do pharmacological tests with calcium channels in single channel reconstitution experiments, giving tools for better identification and understanding of this group of channels.

Channels very similar in their properties (gating, conductance, and selectivity) to the sodium channel results of Hanke et al. were also found in experiments using other preparations, for example, in membrane fragment vesicles from isolated bovine rod outer segment membranes.[72] The physiological relevance of this channel type is unclear, as is the answer to the question of just how far the channel is connected to the physiology of the sodium channel.

Other channels which are voltage dependent and have been reconstituted into planar bilayers for example are a proton-selective channel formed by yeast mitochondria proteolipids,[97] and the voltage-dependent anionic channel (VDAC)[98,99] from *Paramecium aurelia* mitochondrial membranes.

3. Voltage and Ion-Gated Channels

Channels which depend clearly in their kinetics on both the voltage and the concentration of a specific ion present in the aqueous solution, are defined as being voltage- and ion-gated channels.

The most pronounced example of these channels is a Ca^{++}-dependent potassium-selective channel of large unit conductance. Different from most other channels, but comparable to the sodium channel, this channel has been investigated extensively by use of the patch-clamp technique (e.g., in the membranes of cultured rat myoballs and in other membranes).[100,101] Therefore detailed single channel data are available for comparison with the data from reconstitution.

Latorre and co-workers incorporated this channel into decane-containing planar bilayers by fusion of membrane fragment vesicles from rat muscle with the bilayer (Sections II.B.1.a and II.C.2.c).[42,102] After fusion of the vesicles with the planar bilayer a potassium-selective channel of a unit conductance of $\lambda = 290$ pS in 0.2 *M* KCl was found. The kinetic of this channel is strongly dependent on the applied potential and on the concentration of free calcium in the aqueous solution.

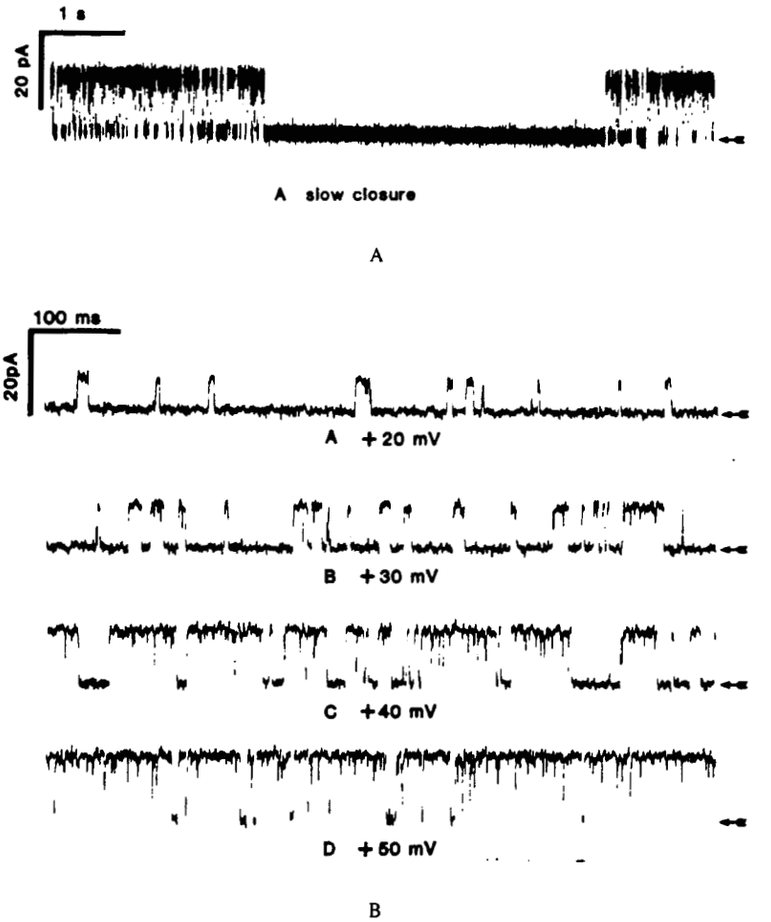


FIGURE 10. After incorporation of membrane fragment vesicles from rat muscle into painted planar bilayers by fusion, a calcium-activated potassium-selective channel of a unit conductance of about 300 pS in 200 m*M* KCl is found. This channel shows a pronounced bursting behavior which is demonstrated in trace a on a compressed time scale. The traces in part (B) were taken with a higher time resolution, so only the inner burst behavior is shown. The traces in (B) were taken at different voltages to demonstrate the strong voltage dependence of the inner burst kinetics of this channel. The traces in part (C) of this figure are comparable to those in part (B). They were taken at different calcium concentrations at a fixed potential to demonstrate the calcium concentration dependence of the inner burst kinetics. In traces (B) and (C) it is clearly demonstrated that the probability of being in the open state within a burst, as well as the open and closed lifetimes within bursts, are strongly voltage and $[Ca^{++}]$ dependent. The same has been shown qualitatively for the bursting behavior itself.[87] (From Moczydlowski, E. and Latorre, R., *J. Gen. Physiol.*, 82, 511, 1983. With copyright permission of the Rockefeller University Press.)

In Figure 10a, a current fluctuation trace of this channel is shown on a compressed time scale and in Figures 10b and 10c traces of current fluctuations of this channel are shown at different potentials and different calcium concentrations on a more expanded time scale. The trace in Figure 10a shows clearly some properties of the gating of this channel. The channel has two distinct conducting states, closed (0 pS) and open (290 pS). The opening events do not occur randomly, but rather in well-separated blocks, usually called bursts.

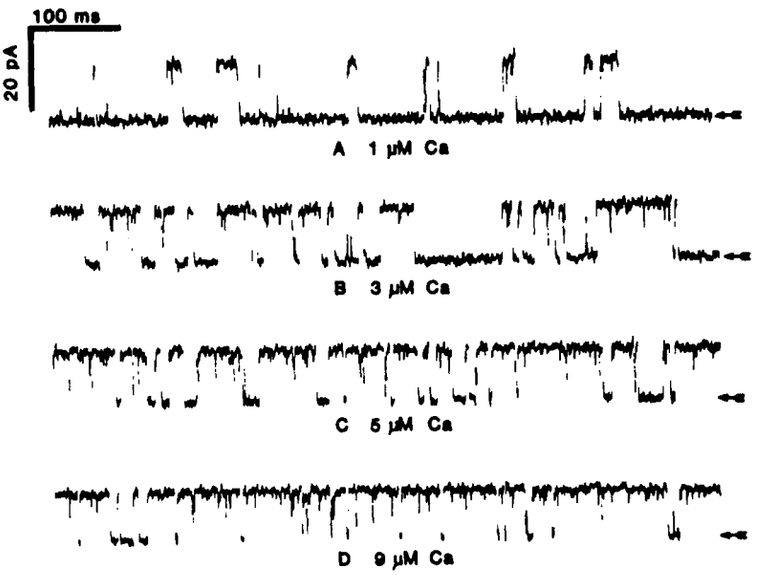


FIGURE 10C.

From the statistical evaluation it is significant that within the burst the events do not originate from more than one channel. Thus, this bursting behavior is a specific property of the Ca^{++}-act. K^+-channel. The bursting can be described by a kinetic process different from that used to describe the inner burst behavior. For each of these processes a probability of being in the open state (or that the burst is on) can be defined. A burst lifetime and an event lifetime can be defined as well as closing lifetimes between events and bursts. In single channel experiments each of the above-specified properties can be investigated in its dependence on voltage and calcium concentration. Until now sufficient data are available for mainly the faster process — the gating behavior within a burst. The probability to be open within a burst Po, the mean open lifetime of events τo, and the mean closed lifetime between event τc are clearly voltage and calcium dependent. In Figure 10b traces of channel fluctuations are shown at different voltages and in Figure 10c, traces of channel fluctuations are shown at different calcium concentrations. The probability of being in the open state within a burst Po is clearly changing as well as the open τo and closed τc lifetimes. Highly positive potentials and high calcium concentrations are opening the channel. The calcium-activated potassium channel is one of the rare examples where data are also available for the kinetic of the bursting process.[103] It has been shown how different divalent cations block the burst kinetics and some data have been presented about the voltage dependence of it. However, even in this case, there is still some difference between what we know about the faster process inside the bursts and what we know about the bursts and clusters of bursts. This is due to the long time which would be necessary to obtain extensive data for the statistical analysis of the bursting, as the bursts do have lifetimes which are orders of magnitude longer than those of the events within bursts.

The same effects as those described above and comparable channel parameters have been found in the patch-clamp measurements with rat myoballs.[100,101] There the channel shows the same bursing behavior and the same kinetic dependencies. The selectivity and the unit conductance are comparable, too. Therefore the channel from patch-clamp experiments and the channel from reconstitution experiments are defined to be the same channel. By direct comparison of the results it can be shown that the properties of this channel are only weakly

affected by the reconstitution procedure. Here it must also be taken into account that larger amounts of organic solvent are present in the reconstitution system.

Another channel of this type found in reconstitution experiments is a chloride-selective channel from Torpedo electric organ membranes. This channel has not been described in other than reconstitution experiments, due to the impossibility of obtaining direct access to the membranes of electric organ cells.

However, to this channel a number of various reconstitution techniques have been applied. First, the channel was found by fusion of membrane fragment vesicles with decane-containing bilayers made from lipid mixtures containing large amounts of PS lipids.[104,105] Then, the channel was investigated by patching large protein-containing vesicles,[32] and it was also found in bilayer experiments using PC lipids below the phase transition temperature (Hanke and Miller[144]) as well as in Montal bilayers containing large amounts of PE lipids above phase transition temperature[21] and in experiments with glass pipettes using the assembly of protein-containing monolayers on the air-water interface of vesicle suspensions (Hanke and Boheim[141]).

In all these experiments the channel showed about the same behavior; thus it can be stated that the properties of this channel are independent of the reconstitution technique used. Furthermore, the properties of this channel must be independent of the composition of the membranes in which it is incorporated.[106]

In Figure 11a a current fluctuation of this channel is shown. Again the channel occurs in bursts. However, in contrast to the calcium-dependent potassium channel, it has two open states of different conductance. Within a burst the channel is fluctuating between a closed state and these two open states (0, 10, and 20 pS in 200 m*M* NaCl). Fluctuations were never found showing only transitions between the closed state and the first open state.

Again, the kinetics of the channel within the burst and the bursting itself can be investigated. It was found that the channel depends upon the voltage applied and the pH of the aqueous solution in its kinetics. The kinetics of the channel within bursts has been investigated in detail.[21] From these experiments a detailed model of the channel gating can be constructed beginning with the idea that within bursts the fluctuation scheme can be described best by the independent fluctuation of two identical subchannels being gated together by the bursting behavior. The probability of each of the subchannels being in the open state (Po) within a burst is shown in Figure 11b depending of the voltage applied and the pH of the aqueous solution. Qualitative data for the bursting behavior itself show that it is voltage and pH dependent, too, but in the opposite direction of the inner burst kinetics.

No physiological data are available about this channel, and nothing is known about its physiological relevance. Due to the lack of toxins binding irreversibly to the channel, the protein forming this ion channel has avoided, until now, any attempt of purification. It has not even been identified in the membrane from which it originates.

Besides the two channels described above, other channels being voltage- and ion-gated have been found in reconstitution experiments. One of them is the bursting channel formed by Phallolysin.[107] Phallolysin is a highly purified partially water-soluble toxin from mushrooms.[108] The voltage and the kinetics of this channel depend on the pH of the aqueous solution, too. The channel formed by Phallolysin is potassium permeable and has only one open conducting state. However, in its voltage and pH dependence it resembles the chloride-selective channel from Torpedo electric organ membranes up to surprising details.

4. Voltage- and Drug-Gated Channels

The ion channels we will discuss in this section are gated by the presence of certain drugs in the aqueous solution. The most pronounced one known from both reconstitution and patch-clamp experiments is the well-known cation-permeable channel formed by the acetylcholine receptor protein,[19,20,22,23] which originates mainly from the postsynaptic mem-

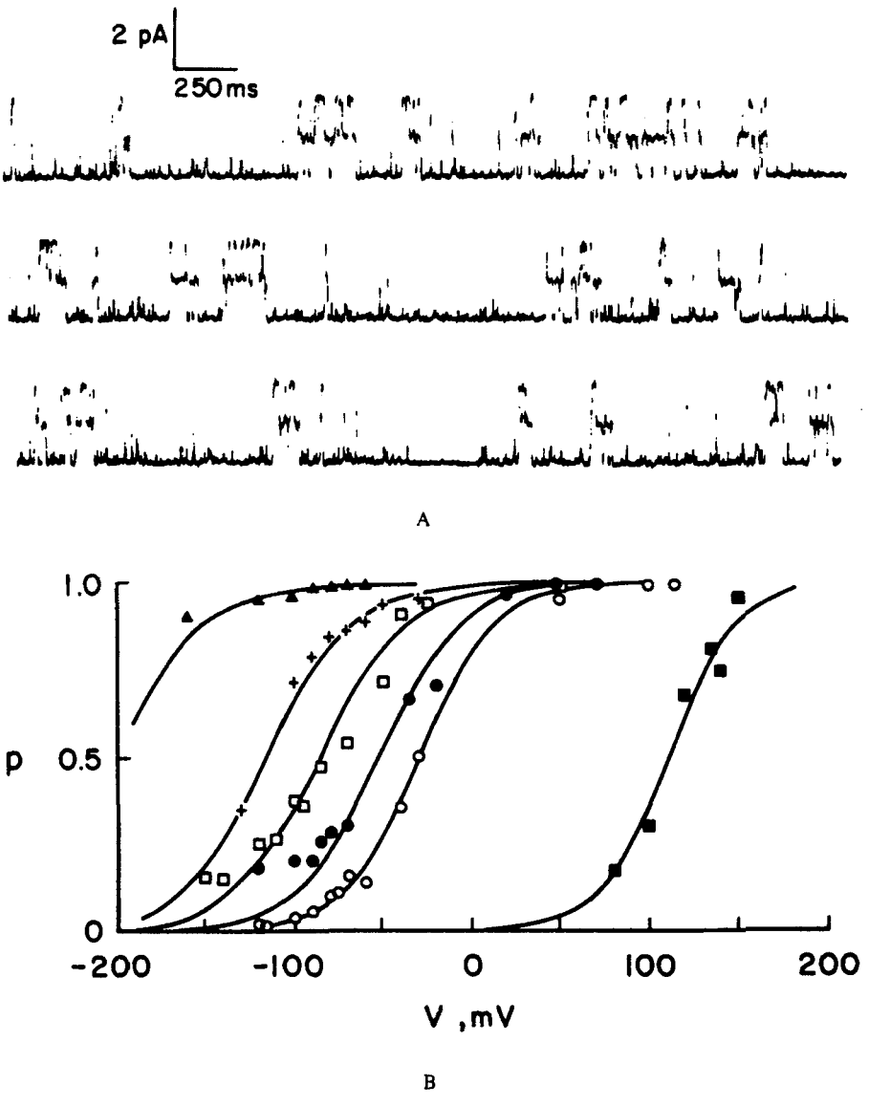


FIGURE 11. Some traces of current fluctuations of the chloride channel from Torpedo electroplax are shown. In 200 m*M* NaCl the channel has a unit conductance of 20 pS in its highest state. The channel clearly fluctuates in bursts. Within the bursts it has two equally spaced open states of 10 and 20 pS under the condition given above. As the two open states of the channel are equally spaced and as it was shown by statistical analysis, the channel can be best described by the independent fluctuation of two identical subchannels within the bursts. The kinetic properties of these subchannels are strongly voltage and proton concentration dependent. This is demonstrated in (B) by the plot of the open state probability vs. the applied voltage at different pH values. (From Hanke, W. and Miller, C., *J. Gen Physiol.*, 82, 25, 1983. With copyright permission of the Rockefeller University Press.)

brane and the membranes of the cells of the electric organs of certain fishes. The drug dependence has been described in detail in electrophysiological experiments. In addition, patch-clamp experiments have been performed describing the channel in single channel experiments,[109,110] using mainly cultured rat myoballs. Therefore sufficient data are available for a comparision of bilayer reconstitution experiments with more physiological experiments.

The acetylcholine-receptor channel has been the subject of extensive biochemical studies. Therefore preparations are available of highly purified protein using the pharmacological properties of it.[111-113] As a consequence, comparison is possible between experiments using membrane fragment vesicles as starting material for reconstitution and experiments using highly purified proteins. These data then can be compared to the electrophysiological data and patch-clamp data.

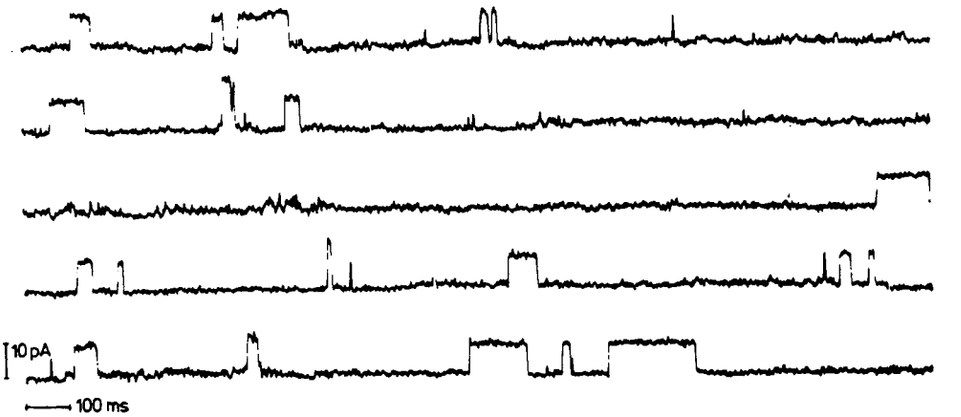
The acetylcholine-receptor protein has been reconstituted by several groups. Schindler reconstituted it by the use of membrane fragment vesicles. Bilayers formed from protein-containing monolayers on the air-water interface of vesicle suspensions were the membranes he used for reconstitution.[23] The behavior of the channel fluctuations in his experiments was about that expected for physiological experiments. Nelson et al.[24] reconstituted the purified acetylcholine receptor protein into planar bilayers using the same technique, and Montal et al.[36] reconstituted the same into bilayers on tip of glass pipettes. In addition Labarca et al.[115,116] and Tank et al.[117] recently presented more detailed data about the reconstitution of the purified receptor protein. From the results of Labarca et al.[115] some more ideas can be taken about the pharmacology of the acetylcholine-receptor channel in bilayer reconstitution experiments, although there are some differences compared to patch-clamp data. The number of agonists to bind to the receptor protein was found to be one by Labarca et al.,[115] whereas from patch-clamp data at least a binding of two agonist molecules was found. Tank et al.[117] showed the possibility of patching large liposomes containing the purified receptor in their membranes. One of the things they demonstrated in more detail was the comparable fast desensitization of the channels, which is well known from patch-clamp experiments. In general the results all groups found indicated that the properties of this protein were not signficantly affected upon purification and reconstitution. This especially was shown by Boheim et al.[19,20] They performed planar bilayer reconstitution experiments with both the purified protein and the protein from membrane fragment vesicles. The technique they used was the reconstitution of the protein by fusion of vesicles with the protein in their membranes with planar lipid bilayers made from PC lipids below the phase transition temperature.[19,20,71] They demonstrated the function of the protein under these conditions and the fact that both protein preparations gave about the same results. The comparison of their results to those from patch-clamp experiments showed good agreement of the data. Furthermore, from the experiments described above, it could be related that the function of the ion channel formed by the acetylcholine-receptor protein depends on the physical state and the composition of the planar bilayers. It has been attempted by other groups to reconstitute this protein into decane-containing bilayers. None of these experiments were successful, thus indicating that the protein is affected by the amount of solvent present in the planar bilayer.

Although a great deal of work has been done with the acetylcholine-receptor protein, there is still a lack of extensive data concerning the single channel parameters and their dependence on voltage and drug concentration. This indicates that there are still problems in the understanding of this protein.

Figure 12 shows traces of single channel current fluctuations included by the acetylcholine receptor protein in planar bilayers in comparison with fluctuations from patch-clamp experiments. All traces were taken in the presence of an agonist, as the channel is not active without. The traces resemble each other even in details as there is the appearance of two classes of channel fluctuation which are different in unit conductance and in the mean event lifetimes.

The dependence of the mean channel lifetimes on the agonist used for activation has been shown, as well as the ability to block the channel by the presence of different toxins.[19,36,114-117]

As pointed out in the section describing the voltage-gated channels, the sodium channel may be included in being a drug-gated channel. It can be opened, for example, by BTX under constant voltage conditions, where it otherwise is not active.



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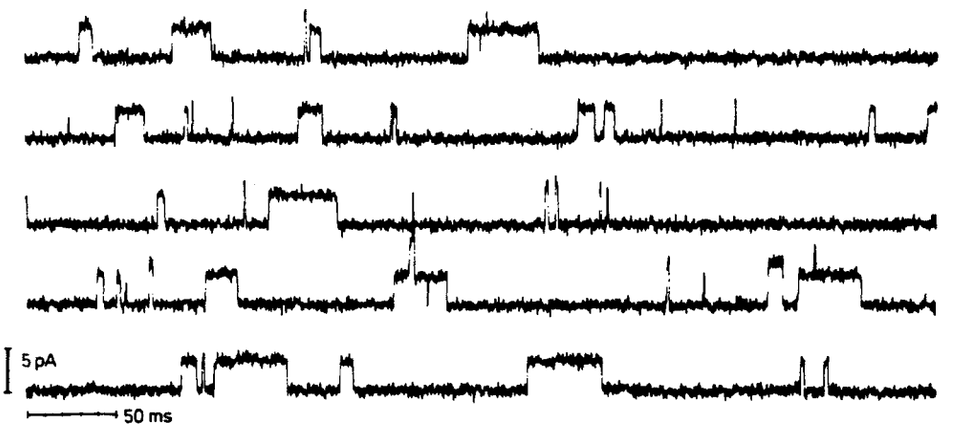


B

FIGURE 12. Part (A) shows a continuous recorded trace of fluctuations in 1,3-SMPC bilayers below Tc after incorporation of acetylcholine receptor protein into the bilayer by fusion of protein containing membrane fragment vesicles from Torpedo electroplax with the bilayer. After addition of 4 μM carbamylcholine, two different channel types were recorded fluctuating in the same membrane. A voltage of -100 mV was applied to the membrane, the aqueous solution contained 1 *M* KCl, and the mean unit conductances of the two channel types were 80 and 125 pS under the given conditions. The larger channel events had clearly shorter lifetimes than the smaller ones. Part (B) shows a recording from a patch-clamp experiment at -70 mV in 140 m*M* NaCl for comparison. The fluctuation scheme of the channels there, which was recorded from myoballs with the presence of 10 μM carbamylcholine, is very similar to that from the reconstitution up to the finer details, as there is the occurrence of two difference channel types: one larger, shorter one and one smaller, longer one. (From Boheim, B., Hanke, W., Methfessel, C., Eibl, H., Kaupp, U. B., Mallicke, A., and Schultz, J. E., in *Transport in Biomembranes*, Antolini, R., et al., Eds., Raven Press, New York, 1982. With copyright permission of Raven Press.)

Not very many results have been presented describing the reconstitution of other voltage- and drug-gated channels. One of the drug-gated channels described in the literature qualitatively is the dopamine receptor from rat corpus striatial.[118,119]

C. General Remarks on Some Properties of Reconstituted Ion Channels

Most of the reconstituted ion channels do have some common properties. The same properties very often have been described for ion channels from patch-clamp experiments. In this section we will discuss some of these interesting findings independent of the channel type with which they were found, due to the fact that they may be of more general importance for all channel types. Furthermore, until now, not too much effort has gone into the investigation of these more common properties among ion channels.

1. Bursting of Channels

As already shown above in some of the examples, a lot of channels show a gating behavior with more than one open and closed state. This is indicated by the occurrence of more than one exponential in the lifetime evaluation of the open and the closed state. The fluctuation scheme found very often is that of single channel events being stringed to bursts. Not all channels show a pronounced bursting, but in a more general theory of channel gating the bursting of channels may play an important role. In addition, it should be pointed out that channels not found to show bursts at some conditions may show bursts at others. The acetylcholine receptor is a good example of this behavior[120] and the sodium channel may also belong to this class of proteins. Detailed theories have already been developed taking into account the bursting behavior of channels. On the basis of this more detailed knowledge of channel gating even the more recent theories of membrane excitation may need some revision.

2. The Appearance of Substates

An earlier and of course simplified view on channel function was that a channel had only open states of defined conductance. Even more simplified is the idea of a channel having only one well-defined open state. Using high-current resolution measurements it was found in reconstitution and in patch-clamp experiments that a lot of channels do have more than one conducting state.[21,106] The chloride-selective channel described above is a good example of a channel having two conducting open states.[21]

Besides the appearance of more than one open state, a good number of channels showed discrete steps present within an open event of a channel. It is difficult to differentiate between the presence of more than one open state and the presence of so-called substates. In Figure 13 two examples are presented of the appearance of substates. The inactivating channel of high conductance from purified TTX binding sodium channel protein[67] has one open conducting state which is by far predominant. However, sometimes the channel opens up only to part of this final value. The obtained steps show always well-defined plateaus, but these are scattered over a wider range of amplitude. The probability of being open in one of these substates is small. In Figure 13b an example of the occurrence of substates is shown for the calcium-activated potassium channel having been discussed above.

The physiological relevance of these substates and of a multistate behavior of channels is unclear. However, such substates were found in patch-clamp experiments, too.[121]

3. Randomly Occurring Changes of Channel Properties

The properties of ion channels are usually taken as being stable with time at steady-state conditions. In evaluating, for example, the kinetic or conductance parameters of a channel, it is necessary to make this assumption. A behavior found for a large number of channels is, however, that random changes can occur in channel parameters.[46,67,72] This has been described, for example, for the calcium activated potassium channel which exhibits just such randomly occurring changes in its kinetics.[42] Figure 14a shows a current fluctuation of this channel. At a defined time the channel spontaneously changes its probability of being in the open state within a burst. In Figure 14b an even more pronounced example of such a

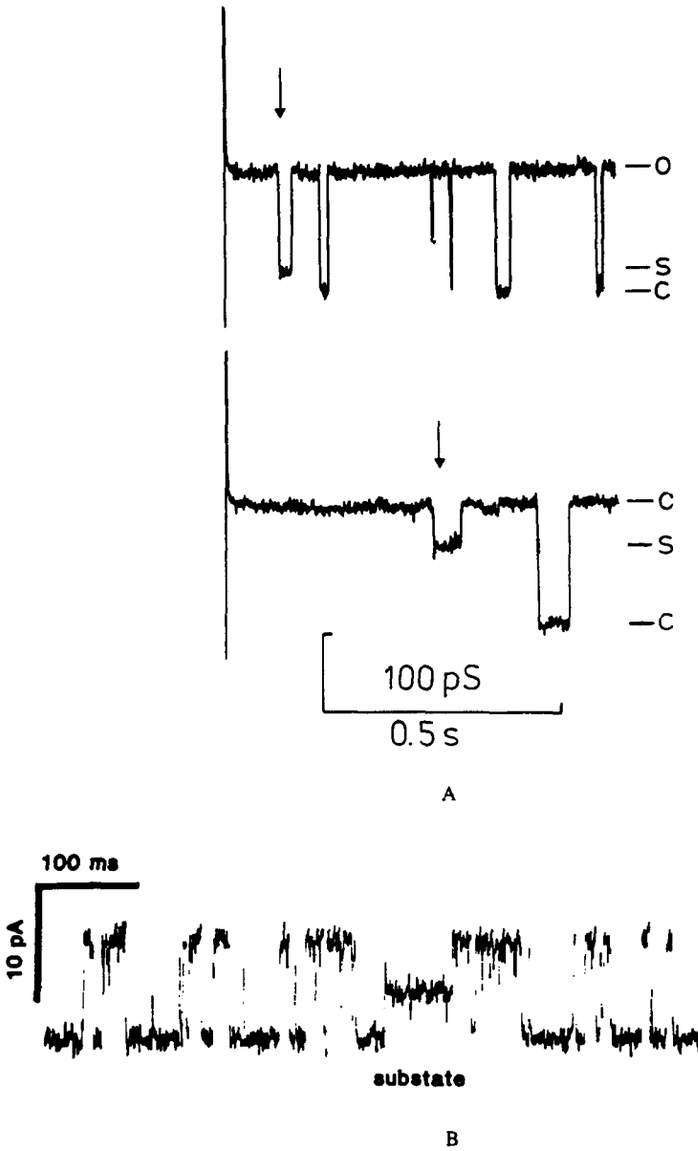
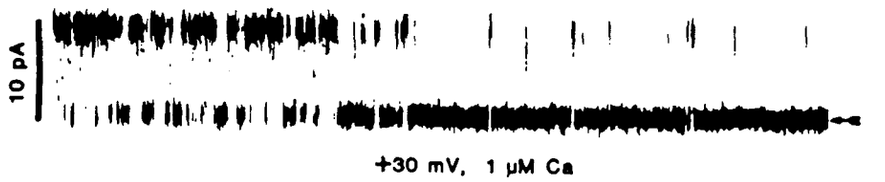


FIGURE 13. Using the activating-inactivating channel from TTX-bound purified protein reconstituted into virtually solvent-free planar lipid bilayers, we demonstrate here the occurrence of substates on some traces (part A). The conditions were the same as given in Figure 9. Each substate of the channel is indicated by an arrow. (B) Shows the occurrence of substates in a current fluctuation of a single calcium-dependent potassium channel. The trace was taken under conditions comparable to those given in Figure 10, using the same preparation. (Part B from Moczydlowski, E. and Latorre, R., *J. Gen. Physiol.*, 82, 511, 1983. With copyright permission of the Rockefeller University Press.)

behavior is shown. The channel of large unit conductance from purified TTX-binding protein not only changes its kinetics at such a randomly occurring event, but also loses its inactivating behavior and in addition changes its conductance (the selectivity of the channel may change at this point, too).[67,72]



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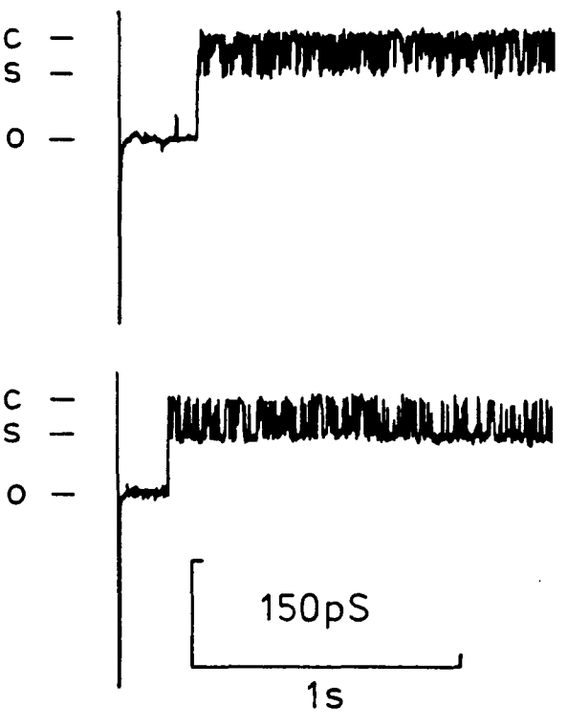

B

FIGURE 14. Random changes may occur in channel kinetics. An example of such a behavior of the calcium-activated potassium channel from rat muscle membranes is given in trace (A). The channel here changes its probability of being in the open state within a burst. In part (B) a more dramatic example of such a behavior is shown. The large inactivating channel from TTX-binding protein not only suddenly changes its kinetic behavior, but in addition changes its unit conductance and selectivity. In addition, in the examples given, the channel loosed its inactivation behavior together with such a change in channel gating. (Part A from Moczydlowski, E. and Latorre, R., *J. Gen. Physiol.*, 82, 511, 1983. With copyright permission of the Rockefeller University Press.)

Until now, no idea has been presented about the role of these randomly occurring changes in channel parameters in the membrane transport processes. Nevertheless, comparable effects have been found in patch-clamp experiments.[122]

4. Solvent Dependence of Channel Properties

It was found that reconstituted channels could be divided into two classes. The first class includes those channels which are independent of the amount of solvent being present in the reconstitution system, having thus parameters not significantly changed by the technique

used for reconstitution.[106] The chloride-selective channel from Torpedo electric organ membranes is a pronounced example of this class of proteins. The second class of proteins is strongly affected by the amount of solvent being used.[106] The acetylcholine-receptor protein may be considered to belong to this class.[19,106]

5. Lipid Dependence of Channel Properties

It is evident that the reconstitution techniques are the only chance to study the lipid dependence of channel properties. However, very little effort has gone into experiments investigating the lipid dependence of channel proteins. For the acetylcholine-receptor protein it has been shown that its function to form ion channels depends on the physical state[19] of bilayers and on the presence of cholesterin[19,23] in bilayers. The effect of the presence of negatively charged lipids on the channel parameters has been demonstrated in some experiments, for example, with the chloride channel[123] or the potassium selective channel from the SR.[124]

D. Molecular Picture of Channel-Formation and Gating Properties

It is almost impossible to develop molecular models of ionic channels from only the data of reconstitution experiments. At least some structural and biochemical data are necessary to do this. For a long time these data were available only for small ion pore forming polypeptides.

The molecular model of the Gramicidin pore has been discussed therefore in detail and is generally accepted. The Gramicidin pore is formed by dimerization of two monomeres, each of which is incorporated into one lipid monolayer of the membrane in helical form. Due to the alternating D-L configuration of the amino acids of the Gramicidin molecule this structure is greatly favored in the membrane.[125] The gating of the pore takes place by the association and dissociation of the two involved monomers which are able to diffuse laterally in the monolayers into which they are incorporated.

Another ionic pore which has been investigated in detail and therefore became a model system for porus ion transport, is that formed by Alamethicin and its analogs. Although the structure of the Alamethicin molecule is known, there are still some problems with the model of the ionic pore formed by it. The following model is supported by the strongest evidence: the Alamethicin molecule forms an α-helix which is incorporated into the bilayer and spans the hydrophobic core of it. The pore itself is formed by aggregation of several molecules which produce a channel of variable diameter bound by a varying number of molecules. This so-called barrel staff model was first presented by Boheim.[126] Additional data led to some modification of the model. The gating of the pore is given by a potentially enforced flip-flop of single Alamethicin molecules within a preformed aggregate. This produces a pore of variable diameter which fluctuates between some different nonintegral states.[127] The potentially enforced flip-flop of single Alamethicin molecules is enabled by the dipol moment of the α-helical part of the molecule.

The model of the Alamethicin pore may be of some more general interest. Although often little is known about the structure of ion channel-forming proteins, there is increasing evidence in many cases that these proteins have large parts of α-helical structures incorporated into the membrane. Due to the large differences in molecular weight between Alamethicin and most of the biological pore forming proteins (Al 2,000 d and biological proteins up to 300,000 d) it is not very likely that the barrel staff model of the Alamethicin pore may play a more general role. The tilting of an aggregate of α-helices may be sufficient, however, to open and close the channel.

Different from many other pore-forming proteins from biological membranes, the acetylcholine-receptor molecule has been the subject of intensive studies and its structure and sequence have been explored. Additional data are available for the channel formed by this

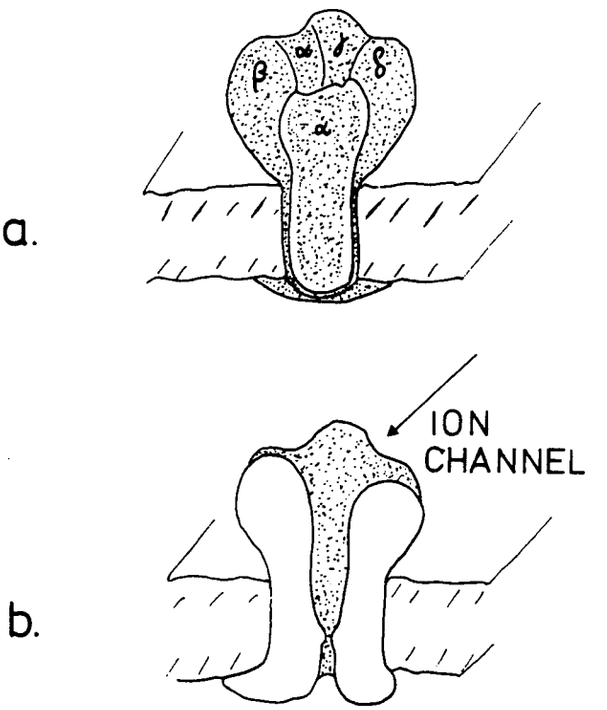

FIGURE 15. Schematic representation of the acetylcholine-receptor channel formed by an aggregate of subunits which are arranged clockwise as shown in part a of the figure. Each of the subunits may form one or more α – helices which are incorporated into the hydrophobic part of the lipid matrix of the membrane. Part of each of the subunits is arranged outside the membrane in a random coil formation. Part b of the figure gives a view into the interior of the ion channel itself. This channel may be opened and closed by a tilting movement of the helical parts of the subunits (details are not shown here). For further information see, for example, Reference 121.

molecule from electron microscopy[128,129] and other techniques.[130] From all that data the following model has been constructed: the molecule is composed from five subunits arranged in the membrane in the clockwise order α,β,α,γ,δ. Each subunit has one or more α-helical parts spanning the hydrophobic core of membrane. Part of each of the subunits is arranged outside the membrane in a random coil structure. In the closed state of the channel the helices are arranged in a form allowing no or only a small pathway through the center around which they are arranged. By a tilting movement this pathway can be made large enough to enable monovalent cations to pass the transmembrane channel formed by this. The molecule can undergo these conformational changes only in the presence of certain agonists, which include acetylcholine. Figure 15 shows a sketch of the molecular model of the acetylcholine-receptor channel. Part a shows the arrangement of the subunits and part b gives a look into the interior of the channel itself.

Another channel which has been investigated in detail in its structure and function is the gap-junction channel.[131] For this channel a detailed model has been developed from data similar to that for the acetylcholine receptor, but without knowing the sequence of the molecule. Nevertheless, a model being very similar to that of the acetylcholine-receptor channel has been established for the gap-junction channel[132] using mainly electron microscope and channel-gating data.

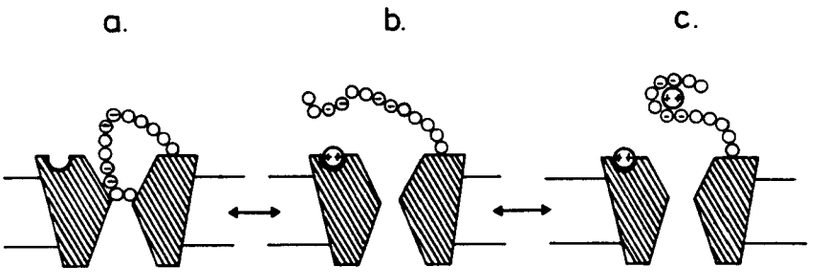

FIGURE 16. Schematic representation of the molecular mechanism of the gating of the calcium-activated potassium channel. The channel is opened and closed by a gate, which can be locked outside the channel itself when calcium is bound to it. The gate can only leave the channel when a calcium ion is bound to the channel-forming part of the molecule. This mechanism is not shown in the figure in more detail. For details see, for example, Reference 41 and 84.

Due to the problems connected with the investigation of the structure of large proteins and the difficulties in making channel models without these data, only rough molecular models exist about ion channels, with the exception, of course, of the examples just mentioned. The calcium-activated potassium channel described above is a good example for this. The sequence and structure of the channel are unknown. Data about the channel are available only from patch-clamp experiments,[100,101] from reconstitution experiments,[42,102,103] and from voltage- and current-clamp experiments;[133] i.e., only kinetic data. From these data a theory was brought forth to explain the gating of the channel. In addition speculations about a possible molecular model describing the activation by calcium have been presented.[100] A gate was postulated which is geometrically separated from the channel itself. The gate closes the channel in the absence of calcium somewhat like a cork. Calcium may bind to the gate due to electrical interaction with negatively charged groups of amino acids in certain positions. The structure of the gate may, for example, be similar to calmodulin in some aspects.[41] When calcium is bound to the gate is is locked outside the channel and cannot close it. A drawing of the model is shown in Figure 16.

A more detailed discussion of molecular models of ion channels and their gating behavior may be the subject of a future review when new data are available about them.

E. Theoretical Aspects of Gating Properties of Ion Channels

In trying to develop a theoretical model for the gating of a certain ion channel, one best starts with extensive single channel data. These data are given in most cases in the form of single ion channel current fluctuation traces. Through visual inspection of such traces it is evident that each channel can adopt several electrically distinct states. One of these states is usually the closed state with a unit conductance of $\lambda = 0$ psec. Under constant experimental conditions a channel fluctuates randomly between the different states it can adopt. According to this fluctuation scheme a probability Pi can be defined to find a channel in the i state. As the channel has to be in one of its possible states at any given time, Equation 1 is given:

$$\sum \text{Pi} = 1 \tag{1}$$

Besides the probability Pi, which an be calculated from the fluctuation by Equation 2

$$\text{Ti/Tges} = \text{Pi} \tag{2}$$

with Ti being the time the channel adopts the i state and Tges being the total time of the fluctuation, the kinetic parameters of the fluctuation being the mean lifetimes of the states

τi, and the unit conductance of each state λi can be calculated. The τi are related to the transition rate constants. These relationships can only be given when all the states a channel can adopt are known. For the simplest two-state channel:

$$C \underset{koc}{\overset{kco}{==}} O \tag{3}$$

Equation 4 holds:

$$1/\tau o = koc \qquad 1/\tau c = kco \tag{4}$$

When a channel has more than two states, these relations become more complicated. Transitions are then possible between any two states. In true experiments the situation usually becomes even more complicated from the fact that not all states are electrically destinct; i.e., there is often more than one state with the conductance 0 pS, which means more than one electrically closed state. Therefore very often Pi, τi, and kij cannot be directly determined. In doing halflg. evaluations of the single-state lifetimes, one can very often find more than one exponential. This indicates that more than one state is present. Sometimes, this situation is evident by visual inspection, when the channel shows a pronounced bursting behavior. In such a case the kinetic parameters of each state can be easily separated due to the fact that the rate constants are, for example, different for orders of magnitude. Usually, some of the processes (for example, the bursting process) are very slow, so that sufficient data are only available for the faster processes since the measuring time would become far too long to obtain the detailed data for the slow process. The kinetic parameters can be calculated in their dependence on experimental parameters such as potential and concentration of certain agonists. This has been done for some channels and gating schemes have been evaluated from these data for the calcium-activated potassium channel[42,100] (for the dependence of the inner burst parameters on the calcium concentration only), for the proton activated chloride channel[21] (for the dependence of the inner burst parameters on the pH only), and for the acetylcholine-receptor channel.[134] Below we will present the models for the calcium-activated potassium channel and for the proton-activated chloride channel, because for these two channels the data are available from reconstitution experiments.

Although a lot of effort has gone into the investigation and reconstitution of the acetylcholine-receptor channel, complete data are not available for the agonist dependence of the channel. Although some detailed models of the channel gating have been presented, using the data of other techniques, there are still controversies about these models. We will not discuss models of this channel here because we want to focus on some examples which are known in detail from reconstitution experiments and which are comparably easy to understand.

From their kinetic data about the calcium-activated potassium channel Moczydlowski and Latorre[42] presented the following gating scheme for that channel within an active burst:

$$C = C_{Ca^{++}} = O_{Ca^{++}} = O_{Ca_2^{++}} \tag{5}$$

This scheme works with the assumption of the binding of two calcium ions to the channel.

Methfessel and Boheim[100] presented a more mechanistic model based on the assumption that the channel can bind one calcium ion in the closed state. Through this a gate, which is otherwise closing the channel, is enabled to leave the channel, and the channel can turn to the open state. When the gate has left the channel it can bind another calcium ion and then cannot close the channel again (see Figure 16). Mathematically this model is equivalent to that of Moczydlowski, but in addition it gives a mechanistic explanation of the gating.

Controversies do exist between the two groups according to the nature of the voltage dependence of the channel gating.

Hanke and Miller[21] calculated a model of the proton activation for the inner burst behavior of the chloride channel. Here the situation is more complicated as the channel has two electrically distinct open states within a burst. These two states, however, are equally spaced. By statistical evaluation of the kinetic data it was shown that inside a burst this channel could be described by the independent switching of two identical subchannels. By this, the calculation can be limited to the description of one subchannel and the total channel behavior can be obtained by calculating all of the data from the binomial distribution of the two independent subchannels. For the single subchannel a molecularity of one was found for the proton binding and the following gating scheme was evaluated:

$$\begin{array}{ccc} C & = & O \\ | & & | \\ CH^+ & = & OH^+ \end{array} \qquad (6)$$

This model is interesting as it is not linear like that for the calcium-activated potassium channel, but a closed circle.

Up to this point we have followed the method of using experimental data in trying to develop a model of the channel gating. As can be seen from the few papers presented so far, this way has not been very successful. Another approach would be to develop a general theoretical model of channel gating and to fit it to each problem separately. Colquhoun and Hawkes[38-40] have started this, but without showing the unhappy researcher how to use the results in explaining experimental data. Methfessel[41] and others used their results to present more practicable solutions. They showed how to create synthetic channel fluctuation traces from a theoretical model and how to compare these to real experiments.

We will not discuss the mathematics used in these theories in detail. The interested reader should use the original papers.[38-41,135,136] To demonstrate, however, the use of these theories we will show a few computer-generated channel fluctuations based on the mathematics mentioned above.

In Figure 17 some traces of simulated channel fluctuations are presented. Part a shows a very simple channel with only two states, open and closed (compare to Figure 7), and a reaction scheme: C = = 0, where C is the closed state, and 0 is the open state. Part b shows a fluctuation of a bursting channel with three states and the reaction scheme: C = = 0 = = I, where I is the closed state between bursts (see Figure 10 for comparision). In part c of Figure 17 the fluctuation of a channel is simulated which undergoes an activation-inactivation sequence after an activating voltage jump (see Figure 9b for comparison).

As can be seen from Figure 17 and as is pointed out in the text, gating of ion channels can be described theoretically quite well. The future problem is now to show by use of experimental data, how far the theories presented describe the reality.

IV. COMPARISION OF RECONSTITUTION TECHNIQUES AND CRITICAL REMARKS ON THEIR USE

Having gone through the literature of 20 years of bilayer experiments and about 15 years of reconstitution we find that several methods have been created to form planar lipid bilayers and to reconstitute ion channel proteins into such bilayers. In addition to this the proteins can be reconstituted into the bilayers of lipid vesicles and investigated there using flux measurements along with other possibilities. We have focused in this article on the reconstitution into planar lipid bilayers and especially on single channel experiments.

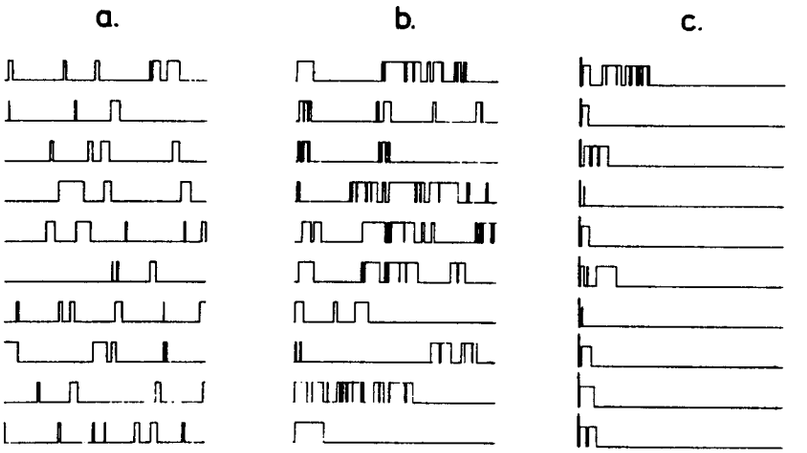

FIGURE 17. This figure shows three groups of computer-simulated traces of channel fluctuations. A scale is not given, as it depends on the setting of the variables of the computer program. Group (a) shows a channel gating according to the very simple scheme C = O. A real ion channel similar in its behavior to this is the cationic channel from fragment vesicles of cilia membranes of *Paramecium tetraurelia*. See Figure 7 for comparison. Group (b) shows a channel with a pronounced bursting behavior similar to that of the calcium-activated potassium channel. The gating scheme is somewhat simpler than for the channel; it is C = O = D (closed, open, desensitized). See Figure 10 for comparison. The last group shows an activating-inactivating channel in its behavior following an activating voltage jump. This is indicated by a capacity transient at the beginning of each trace. The gating scheme is C = O = I (closed, open, inactivated). The behavior of this channel resembles that of the larger channel from reconstituted highly purified STX-binding protein (Figure 9). For details of the calculation of the fluctuation traces and the theoretical basis of it see Reference 41 and 122.

Even in this distinct part of scientific research we face a very special problem. At least some ten or more different channels have been described in the literature in bilayer reconstitution and patch-clamp experiments (single channel experiments). A great deal of isolated data are available concerning all of these channels. The first thing evident from all these data is that not even one channel has been described up to now fully in detail. So, for most of the ion channels known, single channel fluctuations have been presented, with perhaps the selectivity and some details about the kinetics also being published, but that is about all. There are only a few ion channels, which have been investigated in-depth. The acetylcholine-receptor channel has been studied by many different methods including single channel measurements by the patch-clamp technique[109,114,121] and by reconstitution techniques.[19,22-24] The sequence of this protein is known now[130] and a lot of kinetic data are available.[30,36] Reconstitution experiments in vesicles and flux measurements[64] with these vesicles along with bilayer reconstitution measurements[22-24] have not given as much information as one would have expected by the effort gone into these investigations. So even for this ion channel, a great deal more information has to be collected to enable a real understanding of the channel.

Compared to the acetylcholine-receptor channel, the calcium activated potassium channel from different sources has been investigated in more detail.[101,103] It is, together with the chloride channel from Torpedo electroplax,[21] one of the rare examples of ion channels where detailed data are available about one kinetic process (inner burst kinetic)[100] and additionally at least something is known about other kinetic processes (burst kinetic).[103] In addition, the channel has been found in cell membranes by use of the patch-clamp technique.[100,101] However, nothing is known about its structure.

Before looking for other new channels it should therefore be one of the primary goals in future reconstitution experiments to try to understand those ion channels which we have already started to investigate.

Another problem should also be discussed here. When one asks about a more generalized idea of channel formation by proteins and channel gating, he will not find a very satisfactory answer. When looking for things as simple, but important, as lipid dependence of channel parameters[19] or experimental data about the physiological relevance of certain channels, one will find no answer.

Some first attempts have been made in creating more general theories about channel gating.[38-40] Although these theories are mathematically very convincing, the experimental biologist may have a lot of problems in understanding them and, in addition, it may be difficult for him to apply these theories to his special channel. Nevertheless, there is a real need for a general theory of channel formation and channel gating. But to obtain a more generalized understanding of channel properties it is necessary to look for properties which are more common for ion channels in the experiments. This should be included in the problems to be solved in the future and not just to present another hundred new channels.

The answers to all these questions may not necessarily come from bilayer reconstitution experiments. A lot of things can be done much easier through the use of other techniques. For example, single channel data for a number of ion channels from biological membranes can be obtained from patch-clamp experiments as well or better. This especially holds true as some advantages of the bilayer technique are also available for the patch-clamp technique now. For example, methods have been found on how to get access to the inside of the pipette (application of drugs and change of aqueous solution[137].) Other questions, e.g., about the structure of the involved proteins, cannot be answered by the reconstitution technique anyway.

So we should focus on the things which we can really do the best or only by reconstitution of ion channel proteins. To investigate highly purified proteins in reconstitution experiments is one of these things. By using only pure proteins in membranes of defined composition (in the ideal situation, membranes made from one pure lipid), we can create systems which may be understood theoretically not only in their isolated components, but as complete systems. In addition, from such systems questions may be answered about dependencies on certain parameters which are not controllable in other experiments, e.g., the physical state of membranes.[18,19]

After having decided that a certain problem dealing with ion channels may be best solved by using the reconstitution of the ion channel protein into a planar lipid bilayer, the researcher has to decide which special technique of ion channel reconstitution he wants to use. This, of course, depends on the parameters of the ion channel he wants to investigate. There are some general points one should have in mind before starting the experiments.

Of course, the biochemical history of the protein to be reconstituted has to be taken into account when starting to create an assay for the reconstitution of a certain protein. Any method to isolate a protein or to prepare it for reconstitution will affect the protein. As this cannot be avoided, one must try to use procedures to purify the proteins which are as gentle as possible. In addition, it should be demonstrated whenever possible how far the biochemistry affects the properties of the ion channel. With the acetylcholine-receptor this has been done by comparing results from reconstitution experiments with those from patch-clamp and electrophysiological experiments.

After having touched upon the problems of biochemical treatment of ion channel proteins, we can now focus on some of the problems of the reconstitution itself. It is known that any organic solvent does affect proteins. This should be taken into account either by using absolutely solvent-free lipid bilayers (as described above), or by investigating the effect of the solvent used.[106] In general, effects of organic solvents on ion channel proteins are not

of biological relevance, so it seems to be easier directly to use bilayers with as little solvent as possible. It is necessary to keep this problem in mind when using painted bilayers containing large amounts of decane in experiments with large bilayer areas or low capacitance, or to get higher fusion rates in vesicle fusion experiments. The failure in reconstituting the acetylcholine receptor in its functional state into such bilayers is a clear hint.

As the majority of the proteins to be investigated comes in the form of vesicles containing the protein in their lipid bilayers, the most gentle method for reconstitution seems to be to fuse these vesicles with solvent-free planar lipid bilayers.[21] When this is not possible for certain reasons (as there may be too small fusion rates, among others), it is a convincing method to use the self-assembly of lipid protein monolayers on the air-water interface of vesicle suspensions.[16,17,22] The question that has not been answered up to now is in just how far the contact of the protein with the air at the air-water interface affects it. This question is relevant for all ion channel forming proteins, as they must span the monolayer at the air-water interface.

For both techniques, fusion of vesicles and incorporation via the surface, one additional problem must be considered. This is the question about the selective incorporation of one special protein species from a preparation containing more than one protein species.[106] This problem is not given when preparations of purified proteins are used and it may be less important in the method using monolayers on the air-water interface of vesicle suspensions.

The use of patch pipettes for the formation of bilayers may be some help in solving at least a few of the problems demonstrated (amount of solvent and others).

In doing fusion experiments for protein reconstitution, the researcher should always keep in mind that very little is known about the fusion process itself. Only rough tools are available to control the fusion rates, for example. The reproducibility of fusion experiments is not the best for this reason. Very often the problems become even more complicated as there are instabilities in the system which are difficult to control. A better understanding and control of these experimental parameters, especially the fusion rates, should be an important consideration in future reconstitution experiments which make use of the fusion of vesicles with planar bilayers. In addition, this question is of more general interest, for example, in the processes of exocytosis and endocytosis.

The last problem we want to discuss here is that of the reproducibility of reconstitution experiments in general. In the above sections we have sometimes spoken about two component systems, but this seems to be an ideal far away from reality. Very often the experimenter has to fight against contamination in his set-up with unknown things creating artifacts, and it is not always easy to discriminate between artifacts and real events.[138] In addition, every reconstitution experiment has a lot of variables, as there are temperature, potential, curvature of the bilayer, purity of the used salts, and a lot of other different things which are not always possible to control unequivocally. Knowing all these facts, it is somewhat easier to understand that reproducibility in reconstitution experiments means something different from reproducibility in other experiments.

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