Chapter 5

An introduction to the methods available for ion channel reconstitution

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1. Introduction

Reconstitution describes the disassembly of a complex structure; the isolation of one or more of the components of that system and the reassembly of these components into an intelligible, measurable system. When applied to membrane proteins, such as ion-channels, it describes the solubilisation of the membrane, the isolation of the channel protein from the other membrane constituents and the reintroduction of that protein into some form of artificial membrane system which facilitates the measurement of channel function. However, in practice, the term is often applied less rigorously in the study of ion channel function and can be used to describe the incorporation of intact membrane vesicles, including the protein of interest, into artificial membrane systems that allow the properties of the channel to be investigated.

In this chapter I will describe methods that are currently in use for incorporation of both native and purified channel proteins into artificial membranes. The diversity of the subject means that the technical aspects of the various approaches cannot be covered in detail; rather, this chapter is designed to act as an introduction to ion channel reconstitution. Detailed descriptions of experimental protocols can be obtained from the literature cited throughout the chapter.

Ion-channel function can be monitored in a number of ways; by monitoring isotope flux in isolated tissues or membrane vesicles, or by using electrophysiological techniques, such as whole cell voltage-clamp or patch-clamp. What then are the advantages of monitoring channel function following incorporation into some form of artificial membrane?

2. Advantages of ion-channel reconstitution

Not all species of ion-channel are amenable to study by conventional voltage or patch-clamp techniques. Ion-channels found in intracellular membrane systems such

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as the endoplasmic or sarcoplasmic reticulum networks are not readily accessible to extracellular patch electrodes and the elucidation of the single-channel properties of a number of types of channel from these membrane systems has relied heavily on the incorporation of isolated membrane vesicles, and more recently purified channel proteins, into artificial membranes where single-channel properties can be investigated under voltage-clamp conditions (Miller, 1982a; Tomlins *et al.* 1984; Lai and Meissner, 1989; Williams, 1992; Rousseau *et al.* 1988; Bezprozvanny *et al.* 1991).

The transfer of a membrane protein to an artificial membrane of defined phospholipid composition can have a number of advantages for the determination of the biophysical properties of the channel. Ion-channel reconstitution makes possible the investigation of the influence of membrane lipid composition on channel function. For example, the influence on channel function of net membrane surface charge can be investigated by varying the phospholipid composition of the bilayer into which the channel is incorporated (Bell and Miller, 1984; Moczydlowski *et al.* 1985; Coronado and Affolter, 1986).

Similarly, several of the techniques described in this chapter allow the investigator to set and alter the ionic composition of the solutions bathing both faces of the channel protein. Such manipulations are essential for the comprehensive characterization of ion-channel conduction and selectivity (Lindsay *et al.* 1991; Tinker and Williams, 1992; Tinker, Lindsay and Williams, 1992a).

The functional state of purified water-soluble proteins such as cytoplasmic enzymes can be readily determined in solution. However, a functional assay for a purified membrane transport protein, such as an ion-channel, is completely dependent upon the reconstitution of the protein into a membrane which provides a suitably hydrophobic environment for the protein and a barrier through which the channel can catalyze the movement of ions.

3. Methods of ion-channel reconstitution

In this section I will describe methods for the formation of artificial lipid bilayers and the incorporation of ion-channels into these membranes. I will not discuss strategies of membrane vesicle isolation or channel protein purification. These topics are covered in a number of recent reviews (Evans, 1990; Levitski, 1985; Catterall *et al.* 1989; Jones *et al.* 1990; Silvius, 1992). Reviews dealing with channel function following reconstitution are also available (Miller, 1983; Coronado, 1986; Montal, 1987).

The aim of ion-channel reconstitution is to incorporate the channel into an artificial membrane in which its function can be investigated. For practical purposes this means a membrane system in which ion flow through the channel can be studied under voltage-clamp conditions; ideally with good enough resolution to permit the measurement of single-channel open and closed lifetimes. The starting point for such studies is the formation of an artificial planar phospholipid bilayer.

4. Planar phospholipid bilayers

The bilayer is formed across an aperture which links two fluid filled chambers. The size of the aperture and the material of which the aperture is made vary in the techniques to be discussed here, however in all cases bilayers are formed using one of two basic techniques. Readers are advised to consult White (1986) for a detailed description of bilayer formation and the physical properties of planar bilayers.

Spreading from dispersions of phospholipid (painted bilayers)

This method was first described by Mueller and colleagues (Mueller *et al.* 1962; Mueller and Rudin, 1969). The apparatus used in our laboratory for the formation of painted bilayers is shown in Fig. 1. It consists of a block (A) into which is cut an oblong chamber (the *trans* chamber) connected to a second circular chamber which holds the bilayer cup (B). The cup contains a well (volume 500 µl - the *cis* chamber). The face of the cup adjacent to the well is machined to form a thin (approximately 200 µm) septum through which is drilled a hole (200 µm diameter) that, once the cup is located in the block, connects the two chambers. The painted bilayer is formed across this hole.

Bilayers are formed from a dispersion of either one or a mixture of purified phospholipids in a non-polar solvent such as n-decane. Pure phospholipids can be obtained from Avanti Polar Lipids, Alabaster, Alabama 35007, USA. The phospholipid dispersions are made from stock solutions of the required phospholipids

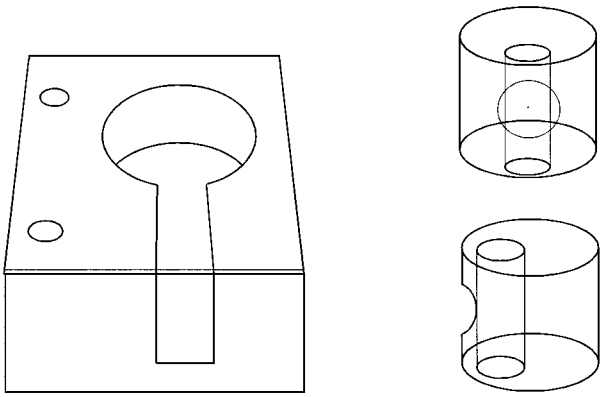

Fig. 1. Experimental chambers for the formation of painted bilayers. (A) Styrene co-polymer block into which is machined the oblong *trans* chamber (volume 1.0 ml) and a well into which the bilayer cup fits. The front face of the block consists of a glass panel through which it is possible to view the hole in the bilayer cup. The two wells at the left of the block are filled with 3 M LiCl and connect the experimental chambers to the head amplifier via agar bridges and silver-silver chloride electrodes. (B) Two views of the styrene co-polymer bilayer cup. The top diagram shows a front view of the cup as it is orientated in the block (A). The lower panel shows the cup from the side and demonstrates the machining of the front wall of the cup to form the septum through which is drilled the hole on which the bilayer is formed. The volume of the *cis* chamber is 0.5 ml.

in chloroform, stored at −80°C. Chloroform is evaporated under a stream of nitrogen and n-decane is added to give the desired final phospholipid concentration (20-50 mM).

Prior to bilayer formation, the hole on which the bilayer is to be formed is "primed" with a small quantity of the phospholipid dispersion. The priming dispersion is allowed to dry, the cup is positioned in the block and both *cis* and *trans* chambers are filled with the desired experimental solution. Additional phospholipid dispersion is then drawn across the hole using a "stick". This implement varies considerably from laboratory to laboratory and may be a small brush, a plastic rod or in some cases an air bubble at the end of a glass capillary.

As described by White (1986), at the outset, the film drawn across the hole will be several μms thick and will be in equilibrium with an annulus (Plateau-Gibbs border) formed as the lipid dispersion "wets" the septum. The film will thin spontaneously to form a bilayer. The primary instigation for thinning comes from Plateau-Gibbs border suction. As the film thins, London-van der Waals attraction between the aqueous phases on either side of the film contributes an additional driving force.

The material from which the cup is made can have a profound influence on the ease of formation and the stability of the planar bilayer. It is possible to manufacture cups from plastics such as Teflon^{R}, polycarbonate and polystyrene. Our experience has led us to use a styrene co-polymer. We have found that this material can be used to produce robust cups on which films thin readily to form stable bilayers. Similar results have been obtained by others using polycarbonate cups (Nelson *et al.* 1984; French *et al.* 1986), although in the past we have found it more difficult to induce film thinning on small holes in this material. Similarly, others have reported that film thinning is difficult to achieve with small holes (100 μm diameter) in Teflon^{R} (Alvarez, 1986).

Thinning of the film can be monitored in one of two ways:-

Observation under reflected light. It is possible to view the phospholipid film using a lens or a low power microscope. On formation, the film appears as a multicoloured structure reminiscent of the pattern seen when a thin layer of oil covers a puddle in the road. The bilayer starts to form at the base of the structure due to the buoyancy of the forming solution (White, 1986) and spreads steadily over the bulk of the hole. As thinning occurs the amount of light reflected from the film decreases until a bimolecular structure is formed (25-50 Å thick), which reflects essentially no light and appears black; hence the name black lipid membrane or BLM.

Monitoring membrane capacitance. Following ion channel incorporation, current flow through the bilayer is monitored using an operational amplifier as a current-voltage converter (Miller, 1982b). The *trans* chamber is clamped at virtual ground whilst the *cis* chamber can be clamped at a desired holding potential relative to ground. An indication of the capacitance of the membrane formed across the hole can be obtained using a low frequency (1 Hz), low amplitude (5-20 mV peak to peak) square wave. The application of this wave form to the system before the lipid film is applied to the hole results in large, saturating oscillations in current; the solutions in the *cis* and *trans* chambers are electrically coupled through the hole. On application

of the lipid film to the septum, the hole is blocked by the forming solution and a small deflection from zero current is seen with each voltage clamp transition. As the film begins to thin the current deflection will increase in amplitude as the capacitance of the film increases. The final capacitance of a painted bilayer should be in the region of 0.4 μF/cm^2 (Alvarez, 1986). As hole size and geometry will vary from cup to cup, bilayer formation, as adjudged by capacitance, is often determined empirically; the operator learns the amplitude of the capacitance spike that will allow good channel incorporation and hence signifies stable bilayer formation. The height of the spike will be dependent upon the rise time of the square wave and the capacitance of the membrane.

What to do if the film will not thin

In practice we have all encountered times when application of the forming solution to the hole results in a blob of lipid which will not thin spontaneously to form a bilayer. In my experience this situation usually arises when the hole is surrounded with too large a quantity of forming solution. Under these conditions it is sometimes possible to induce the film to thin by applying a large voltage pulse to the system (e.g. ±100 mV) or by repeated painting of the hole with a clean painting stick. However, the best solution to this problem is to remove the cup and clean the septum before re-priming.

Chamber and solution preparation

As with all single-channel monitoring techniques, it is very important that the experimental chambers of the bilayer system be kept clean. The polystyrene chambers used in our laboratory are cleaned with a household dish washing detergent and are rinsed thoroughly under running water and dried before use. Similarly, chambers can be cleaned with methanol and again dried before use. Teflon^R chambers can be cleaned in sodium dichromate-sulphuric acid as described by Alvarez (1986).

All solutions used in the bilayer system should be filtered before use, we routinely make experimental solutions with deionized water and pass them through 0.45 μm diameter pore Millipore filters before use.

5. Forming bilayers from monolayers (folded bilayers, bilayers on patch pipettes)

An alternative procedure for the formation of a planar phospholipid bilayer involves the apposition of two phospholipid monolayers formed at the interface of an aqueous solution and the air (White, 1986). This method has been adopted by some workers because the resultant bilayer contains somewhat less solvent than the equivalent bilayer formed from alkane dispersions of phospholipids by the painting method described above.

Monolayers can be formed by applying phospholipids in a volatile solvent such as pentane, chloroform or hexane to the surface of an aqueous solution; the solvent

evaporates in minutes leaving a phospholipid monolayer at the air-solution interface (Montal and Mueller, 1972; Coronado and Latorre, 1983). Alternatively, monolayers can be formed by allowing phospholipid liposomes or mixtures of liposomes and native membrane vesicles to equilibrate with a monolayer at an air-water interface (Schindler, 1980; Schindler and Quast, 1980; Nelson *et al.* 1980). The latter method leads to the production of monolayers containing native membrane proteins including ion channels. These channels may then be incorporated directly into the membrane on bilayer formation (Schindler and Quast, 1980; Nelson *et al.* 1980; Suarez-Isla *et al.* 1983; Montal *et al.* 1986). Once a monolayer has been formed, there are two standard methods for producing a phospholipid bilayer.

(1) Monolayer folding

As is implied from its name, this method involves the folding together of two monolayers to form a planar bilayer. A brief outline of the method will be given here and interested readers are directed towards the following references for more detailed accounts (Schindler, 1980; Schindler and Quast, 1980; Nelson *et al.* 1980; Montal *et al.* 1986).

The apparatus used for bilayer formation via monolayer folding is in many respects similar to that used in the production of painted bilayers in that it involves two chambers separated by a septum containing a hole. A major difference exists in that the septum used for monolayer folding is not of rigid plastic but is composed of a very thin (10-25 μm) Teflon^R membrane supported between two thicker Teflon^R O-rings.

Holes can be formed in the septum by punching with a hypodermic needle. The tapered end of the needle should be removed and the level end sharpened either mechanically using fine abrasive paper or electrically by etching in 5 M HCl (Montal *et al.* 1986). Alternatively, holes can be produced with an electrical spark from a car ignition coil (Hartshorne *et al.* 1986). We have found this technique to be very useful; a small defect in the Teflon^R membrane is created with a needle and the Teflon^R can then be melted by the spark. The size of the hole produced in the membrane can be increased by discharging additional sparks across the membrane. With practice, it is possible to routinely produce holes with a smooth perimeter and diameters in the range 30-200 μm.

For bilayer construction, the Teflon^R septum is clamped between two chambers (Fig. 2; see Montal *et al.* 1986 for details of chamber design). The hole on which the bilayer is to be constructed is then primed with 0.5% (v/v) hexadecane in hexane (Schindler and Quast, 1980; Montal *et al.* 1986). Once the priming solution has dried, the desired experimental solution is added to both chambers to a level below the hole in the septum, and a phospholipid monolayer is formed at the solution-air interface using one of the methods described above. The bilayer is formed by increasing the level of the solution, first in one and then in the other chamber so that each monolayer is raised to cover the hole (Fig. 3). Formation of the bilayer is monitored by measuring capacitance; the final membrane should have a capacitance of approximately 0.8 μF/cm^2 (White, 1986; Montal *et al.* 1986).

The production of planar phospholipid bilayers by folding monolayers offers the

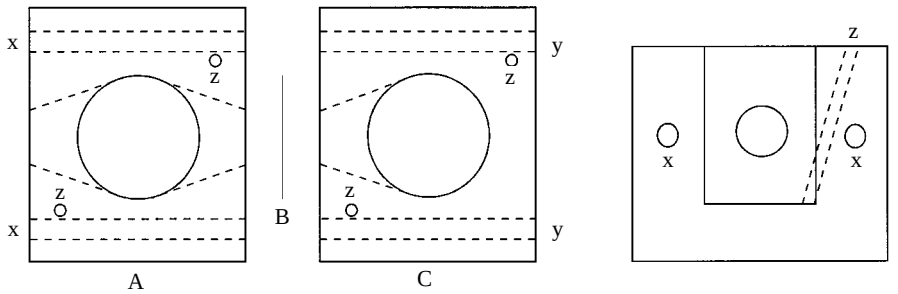

Fig. 2. Experimental chambers for the formation of folded planar phospholipid bilayers (based on a design provided by Montal *et al.* 1986.) Readers are advised to consult this reference for chamber dimensions). A hole is formed in a thin Teflon^R film (B) as described in the text. This film is clamped between two Teflon^R blocks A and C (viewed from the top in the left panel) with bolts that pass through the holes x-y. Each block contains a well which connects with the face of the block in contact with the film. The right panel shows a front view of the chamber. Solutions can be added and raised through ports in the blocks (z). Similar ports are used to connect the experimental chambers to the head amplifiers via salt bridges and silver-silver chloride electrodes.

possibility of making small membranes. I have found it difficult to routinely make painted bilayers on holes with a diameter of much less than 100 μm; with holes of this size it is very difficult to achieve reproducible film thinning. This should not be a problem with monolayer folding. Decreasing the surface area of the bilayer will increase the mechanical stability of the membrane and hence decrease the

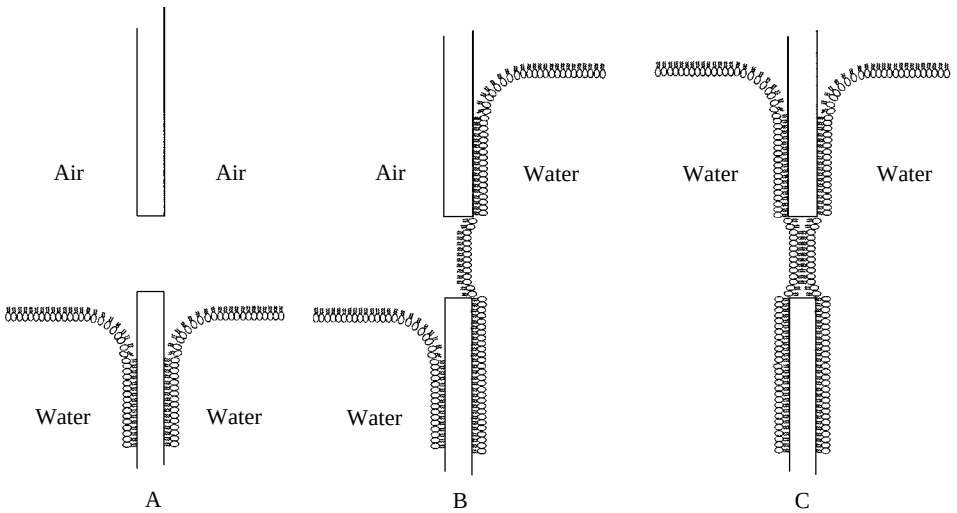

Fig. 3. Cartoon showing the formation of a bilayer on a Teflon^R septum from preformed phospholipid monolayers. Monolayers are formed at the interface of an aqueous solution (water) and the air, using one of the methods described in the text. The bilayer is formed by the successive raising of the level of the solution in the chambers on either side of the septum (A-C). Channel proteins may be incorporated into the bilayer if they are present in either of the monolayers (see text). The septum, hole and phospholipids are not drawn to scale.

background noise of the system. The apposition of two monolayers to form a bilayer also allows the experimenter to construct asymmetric bilayers, for example one monolayer could be formed from an essentially uncharged phospholipid such as phosphatidyl ethanolamine whilst the other might contain a high proportion of a negatively charged phospholipid such as phosphatidyl serine. As stated at the beginning of this section, bilayers constructed from folded monolayers will contain somewhat less solvent than those cast from dispersions of phospholipids in a solvent. However, folded bilayers will not form in the absence of a hydrophobic environment for the formation of the annulus (White, 1986). An alternative method for the formation of bilayers from preformed phospholipid monolayers does permit the construction of truly solvent-free bilayers.

(2) Bilayers on patch-pipettes

The formation of planar bilayers on the end of patch pipettes was introduced by Wilmsen and colleagues (Wilmsen *et al.* 1983; Hanke *et al.* 1984) and has been used by a number of groups to investigate both native membrane channels and purified channel proteins (Suarez-Isla *et al.* 1983; Coronado and Latorre, 1983; Coronado, 1985; Ewald *et al.* 1985; Montal *et al.* 1986). The use of this method appears to have declined in recent years, however, I will discuss it here as it provides a method for the production of small solvent-free bilayers which may be required for particular reconstitution applications.

Bilayers are formed at the end of conventional patch-clamp pipettes (Sakmann and Neher, 1983; Corey and Stevens, 1983) with tip diameters in the range 0.5-5 μm either with or without fire polishing (Montal *et al.* 1986; Suarez-Isla *et al.* 1983; Coronado and Latorre, 1983). The tip of the pipette is immersed in the desired experimental solution in a compartment of a multi-well disposable tray (volume approximately 0.5 ml) and a phospholipid monolayer formed at the air-water interface using one of the methods described above (Fig. 4). A portion of the monolayer is transferred to the pipette tip by raising the pipette into the air. The polar head groups of the phospholipids orientate so that they interact with the aqueous pipette-filling solution and the glass wall of the pipette. The hydrocarbon chains of the molecules face the air. A bilayer is constructed by re-immersion of the pipette in the bath solution. As the tip of the pipette crosses the monolayer at the air-solution interface a second region of monolayer interacts with the monolayer in the pipette to form a bilayer. Bilayers can be formed from a range of purified phospholipids, however seal formation with phospholipids bearing a net negative charge may require the presence of divalent cations in the pipette and bath solutions (Coronado, 1985). If monolayers are created from suspensions of native membrane vesicles a certain proportion of bilayers formed using this method will contain channel proteins (Montal *et al.* 1986). As with all bilayer formation protocols, particular care must be taken to ensure that the pipette glass is clean and that all solutions are filtered before use. It may be desirable to use pipettes and experimental baths only once.

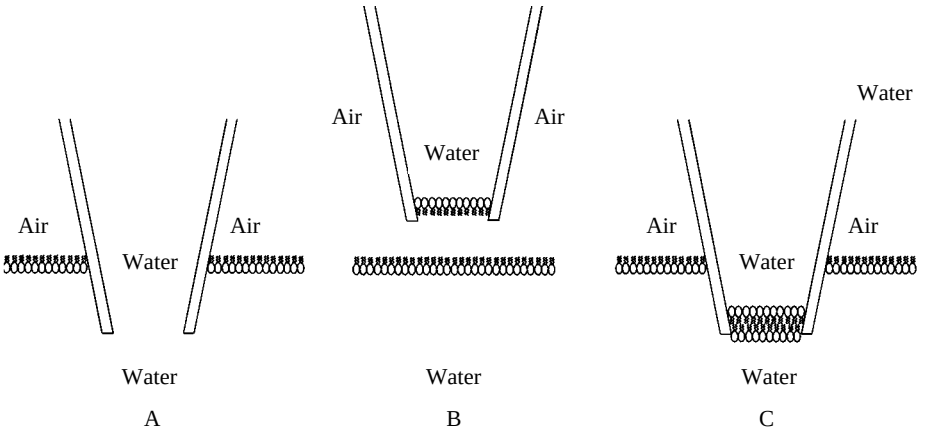

Fig. 4. Cartoon showing the formation of a bilayer from a phospholipid monolayer at the tip of a patch pipette. A bilayer is formed by the transfer of first one and then a second phospholipid monolayer to the pipette tip (A-C; see text for details). Channel proteins may be incorporated into the bilayer during formation if they are present in the original monolayer (see text). The pipette and phospholipids are not drawn to scale.

6. The incorporation of ion channels into planar phospholipid bilayers

The earlier sections of this chapter have provided an introduction to the methods currently available for the formation of planar phospholipid bilayers. I cannot emphasise too strongly the importance of the bilayer in the overall success or failure of an ion channel reconstitution experiment. If the bilayer is sub-standard, that is if it will not thin, or if the bilayer is leaky or unstable, there is absolutely no point in attempting to incorporate ion channels. No matter which of the above approaches is adopted to form the bilayer the resulting membrane must provide a stable, electrically quiet environment for the channel under investigation. Detailed studies of channel conduction or gating often take considerable periods of time, possibly up to an hour. If sufficient care and attention has been taken during bilayer formation, the stability of the bilayer should not be a limiting factor in experiments of this kind.

Some of the methods of bilayer formation described above offer the possibility of incorporating either native or purified channel proteins into the bilayer during formation. However in most cases, following the formation of a stable bilayer, channel proteins must be introduced into the membrane. Whilst there are recent reports describing the incorporation of purified ryanodine receptor-channel proteins into bilayers from detergent solutions (Imagawa *et al.* 1987; Lai *et al.* 1988; Smith *et al.* 1988; Anderson *et al.* 1989), the standard method for the incorporation of both native and purified channel proteins into pre-formed planar lipid bilayers involves the fusion of a channel-containing membrane vesicle with the bilayer; a procedure first described by Chris Miller in his studies of the sarcoplasmic reticulum K^+-selective channel (Miller and Racker, 1976; Miller, 1978).

Native membrane vesicles or proteo-liposomes containing purified channel proteins can be incorporated into planar phospholipid bilayers formed either by spreading phospholipid dispersions or from monolayers on Teflon^{R} partitions or patch pipettes. The broad rules governing fusion are believed to be the same in all cases, although optimal conditions may vary slightly (see for example Cohen, 1986). Much of our understanding of the principles underlying vesicle-bilayer fusion has been derived from studies employing phospholipid vesicles in which fusion has been monitored either by following the transfer of vesicular contents across the planar bilayer (Zimmerberg *et al.* 1980; Woodbury and Hall, 1988a,b; Niles and Cohen, 1987) or by the incorporation of reconstituted VDAC or porin channels into the bilayer (Cohen *et al.* 1980; Woodbury and Hall, 1988a).

Fusion of membrane vesicles with a planar phospholipid bilayer is preceded by the development of a pre-fusion state in which the membrane vesicles become closely associated with, or bound to, the planar bilayer (Cohen, 1986). If either the planar bilayer or membrane vesicle contain a proportion of negatively charged phospholipids, the occurrence of the pre-fusion state can be encouraged by the inclusion of millimolar concentrations of divalent or trivalent cations in the experimental solutions (Cohen, 1986; Hanke, 1986).

Membrane vesicles in pre-fusion association with a planar bilayer will only fuse with the bilayer if they are induced to swell (Finkelstein *et al.* 1986). Vesicle swelling is most commonly induced by forming an osmotic gradient across the bilayer so that the osmotic pressure of the solution in the chamber to which the membrane vesicles are added (*cis*), is greater than that of the solution on the other side of the bilayer (*trans*). Under these conditions water will flow from the *trans* chamber to the *cis* chamber; some of this water will enter the membrane vesicles bound to the bilayer in the pre-fusion state. These vesicles will swell and some will burst leading to a coalescence of the vesicle with the bilayer (Fig. 5).

The osmotic strength of the *cis* solution should be raised using a solute that will readily cross the vesicle membrane and hence increase the osmotic pressure in the vesicle lumen. At the same time the substance should not be so permeant in the bilayer that the osmotic gradient is dissipated. In practice, glycerol and urea are efficient in stimulating vesicle fusion whilst more permeant substance such as ethylene glycol and formamide are less effective (Cohen, 1986). Osmotic gradients created with salt solutions will induce vesicle swelling if the vesicle contains channels permeable to one or both of the ions (Cohen, 1986). The hydrostatic pressure developed in the vesicle induces fusion (Niles *et al.* 1989), therefore the vesicle lumen should ideally also be hyperosmotic to the *cis* solution, this is often achieved by making or storing the membrane vesicles in concentrated sucrose solutions (0.3-1.0 M).

The efficiency of vesicle-bilayer fusion can also be influenced by other factors. The greater the surface area of the bilayer the more likely it is that membrane vesicles will come into pre-fusion contact and hence the greater the likelihood of fusion. The probability of vesicle fusion with a bilayer at the tip of a patch pipette may be so low as to make it impractical. The density of vesicles in the vicinity of the bilayer can be

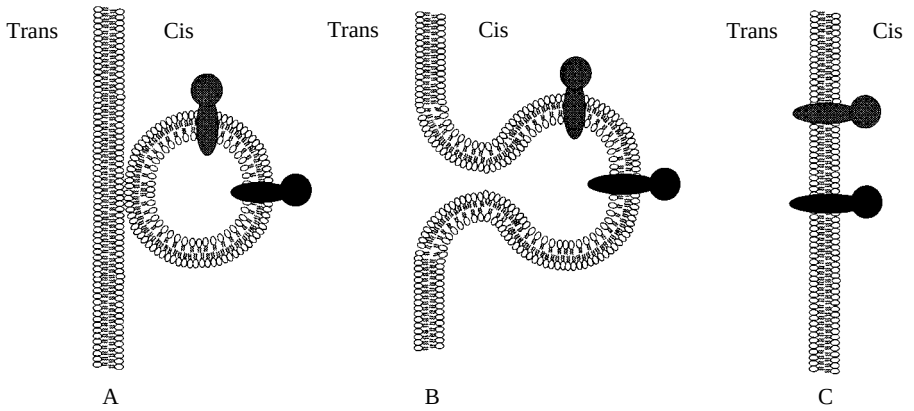

Fig. 5. The fusion of a membrane vesicle with a planar phospholipid bilayer. (A) The pre-fusion state. Membrane vesicles associate with, or bind to the planar phospholipid bilayer. (B) The establishment of an osmotic gradient across the bilayer induces the vesicle to swell, burst and incorporate with the bilayer. (C) Following fusion, the channel proteins (shaded and black areas) are aligned such that the face of the protein protruding from the vesicle faces the chamber to which the vesicles were added (*cis*), whilst the luminal face of the channel faces the other (*trans)* chamber. Not drawn to scale.

increased by adding them to the solution in the pipette rather than to the much greater volume of solution in the bath (Hanke *et al.* 1984). The fusion rate may also be influenced by the phospholipid composition of the bilayer, the presence of divalent cations, the size of the osmotic gradient, vesicle concentration and stirring (Labarca *et al.* 1980; Hanke, 1986; Cohen, 1986). It is sensible to employ a range of fusion conditions when attempting to investigate the channel content of a new vesicle population.

A general principle to emerge from the investigations outlined above, is that vesicles will fuse with planar bilayers in the presence of an osmotic gradient if the vesicle contains a permeability pathway for the solute; vesicles containing channels fuse more readily than channel-free vesicles (Woodbury and Hall, 1988b; Cohen *et al.* 1989). Woodbury and Miller (1990) have recently described a method for maximising vesicle fusion with planar bilayers in which nystatin is incorporated into membrane vesicles in the presence of ergosterol. Nystatin provides a weakly anion-selective permeability pathway in all vesicles so that they readily fuse in the presence of a salt gradient. Nystatin only forms functional conduction pathways in the presence of ergosterol, therefore if the bilayer into which the vesicles incorporate contains no ergosterol, fusion is marked by a transient increase in conductance, which decays as the ergosterol associated with the nystatin in the vesicle dissipates into the bulk of the bilayer. Using this method it is possible to assess the variety of channel species in a vesicle population and to determine the density of ion channels in a preparation of vesicles.

Having established the optimal conditions for vesicle fusion, the investigator is often faced with the problem of how to limit fusion. Studies of channel gating kinetics require the presence of a single channel in the bilayer and so it is important that the

rate of vesicle fusion can be controlled and stopped at the appropriate point. In practice this can be done in a number of ways. If divalent cations are used to encourage fusion with negatively charged bilayers, chelation of the cation dramatically slows incorporation. Similarly, the dissipation of the osmotic gradient across the bilayer will eliminate fusion. This can be achieved either by increasing the osmotic strength of the *trans* chamber or by perfusing the *cis* chamber with a solution of lower osmotic strength. This method has the added advantage that unfused vesicles are removed from the solution.

As vesicles fuse, channel proteins will incorporate into the bilayer with an orientation that is dependent upon their orientation in the vesicle (Fig. 5). In other words, if the sample is made up of populations of vesicles with mixed orientation, or if individual vesicles contain proteins in both possible orientations, then both orientation of channel are likely, or at least possible, in the bilayer. A number of channel properties are side-specific, this is obviously so for agonist-activated channels such as the acetylcholine receptor, the plasmalemmal Ca^{2+}-activated K^+ channel or the Ca^{2+}-activated Ca^{2+}-release channel of muscle sarcoplasmic reticulum where the agonist binding site is located on a specific face of the channel protein. However, a number of channel conduction properties are also asymmetric, for example, it is common for blocking ions to have access to the conduction pathway from only one side of the channel (Tinker *et al.* 1992b,c). Therefore it is important to consider, and if possible to monitor, channel orientation following vesicle fusion with a planar bilayer. Fortunately, many of the channels that have been studied in bilayers, for example the voltage-dependent Na^+ channel (Moczydlowski *et al.* 1984), the skeletal muscle sarcolemmal Ca^{2+}-activated K^+ channel (Latorre, 1986) and the K^+ channel and Ca^{2+}-activated Ca^{2+}-release channel of sarcoplasmic reticulum (Miller and Rosenberg, 1979; Ashley and Williams, 1990), occur in isolated membrane vesicle populations which have a fixed orientation. As a result the channels incorporate into the bilayer with a defined alignment. Smooth muscle sarcolemmal vesicles appear to be randomly orientated and consequently Ca^{2+}-activated K^+ channels from this source incorporate into bilayers with random orientation (Latorre, 1986).

7. Incorporation of ion channels into liposomes suitable for patch-clamping

The final approach to be discussed in this chapter involves the incorporation of either native or purified channel proteins into small unilamellar liposomes and the transformation of these liposomes into structures suitable for conventional patch-clamp analysis.

Native membrane vesicles, isolated by differential or density gradient centrifugation following tissue homogenisation, are too small to patch-clamp (diameter 0.1-1.0 μm). Small unilamellar proteo-liposomes into which purified channel proteins are reconstituted by detergent removal are of a similar size. The size of these vesicles can be increased using either of the following methods.

Freeze-thaw

The formation of large liposomes by the successive freezing and thawing of small unilamellar vesicles was first demonstrated by Kasahara and Hinkle (1977). The use of this procedure to form channel-containing liposomes suitable for patch-clamping was introduced by Tank and Miller (1982, 1983). The method has been used to study chloride-selective channels from native membranes of *Torpedo* electroplax (Tank and Miller, 1982), the K^+-selective channel of native sarcoplasmic reticulum membranes (Tomlins and Williams, 1986), the purified voltage-dependent Na^+ channel (Agnew *et al.* 1986) and the purified acetylcholine receptor-channel from *Torpedo* (Tank *et al.* 1983).

Native membrane channel proteins are solubilised with a suitable detergent and separated from unsolubilised material by centrifugation. The solubilised membrane components or, where appropriate, the purified channel proteins are then incorporated into proteoliposomes. Solubilised proteins are mixed with excess phospholipid and the detergent removed. In the case of cholate, the detergent used in the studies quoted above, this is easily achieved by dialysis; other detergents may require different procedures (Jones *et al.* 1990). The channel-containing small unilamellar liposomes are then transformed into large liposomes by freeze-thaw. An aliquot of the sample is frozen and then allowed to thaw. Freezing can either be carried out using liquid nitrogen or alternatively the sample can be frozen at –80°C; the sample is allowed to thaw either on ice or at room temperature. Following this procedure, the initially clear suspension of small unilamellar proteoliposomes becomes turbid as the result of the production of larger multilamellar structures (Tank and Miller, 1983). The size of these structures may be increased still further by additional cycles of freeze-thaw and it is possible to produce structures with diameters in the range 10-30 μm. Freeze-thaw is believed to produce large membrane structures as the result of vesicle breakage and re-sealing induced by the creation and breakdown of ice crystals. The procedure will not work if the solution in which the vesicles are suspended contains any cryo-protectant; as little as 10 mM sucrose is sufficient to prevent the production of large patch-clampable structures (Tank and Miller, 1983).

Dehydration-rehydration

An alternative method for the production of large proteoliposomes suitable for patch-clamp investigation was described by Criado and Keller (1987) and has been used by them and others to monitor channel activity in a range of native and purified channel species (Keller *et al.* 1988; Riquelme *et al.* 1990a,b). As with freeze-thaw, the initial stage in this procedure as described by Criado and Keller is the solubilisation of the channel protein and its incorporation into small unilamellar vesicles. These authors employed Chaps (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate) to solubilise membrane preparations ranging from skeletal muscle sarcoplasmic reticulum to chloroplast envelopes. Solubilised channel proteins were then incorporated into small unilamellar vesicles by dialysis in the presence of exogenous lipids (Keller *et al.* 1988). Aliquots of the resulting proteoliposomes were

sedimented by centrifugation and the pellet resuspended in a small volume of 10% Mops buffer (pH 7.4) containing 5% (w/v) ethylene glycol. A drop of this suspension was applied to a glass slide and dehydrated at 4°C in a desiccator over $CaCl_2$. The ethylene glycol in the suspension prevents complete dehydration. With a starting volume of 20 μl a period of 3 hours dehydration was allowed before rehydration was initiated by the addition of 20 μl of 100 mM KCl, or other experimental solution, to the partially dehydrated suspension. Rehydration was allowed to continue overnight at 4°C. At the end of this period large multilamellar liposomes ranging in diameter from five to a few hundred μm were seen at the edges of the rehydrated film.

A variation of this technique has been used by Riquelme *et al.* (1990a). These authors used membrane vesicles prepared from *Torpedo* electroplax, from which peripheral proteins had been removed by alkaline extraction, to monitor acetylcholine receptor-channel activity. These native membrane vesicles were added to phospholipid liposomes and the mixture subjected to partial dehydration and rehydration as described above. The same group have used dehydration-rehydration to monitor single-channel events from purified glycine receptors (Riquelme *et al.* 1990b).

Patch-clamping large proteoliposomes

Irrespective of the method used to form proteoliposomes, single-channel properties of channels incorporated into these structures can be investigated using conventional patch-clamp procedures. Using fire polished pipettes (tip resistance 5-20 GΩ), seals (10-200 GΩ) are readily obtained (Tank and Miller, 1983; Tomlins and Williams, 1986; Keller *et al.* 1988; Riquelme *et al.* 1990a). Channel activity is best monitored following excision of the patch from the proteoliposome (Tank and Miller, 1983).

8. Which method should be used?

Clearly there are a number of methods available to a worker wishing to investigate the properties of an ion channel in a reconstituted system. Before embarking on a study using any of these approaches it is worth spending some time considering which is the most appropriate for the task. Some factors worthy of consideration are:-

(1) *Ease of use and reliability.* It can probably be argued that the easiest and most reliable method of ion channel reconstitution is the one with which any particular investigator is most familiar. A brief survey of the literature would suggest that most workers favour planar lipid bilayers formed on holes in partitions, and of these the majority involve bilayers spread from a dispersion of phospholipids in n-decane (painted bilayers). In my experience, painted bilayers are the easiest to make and will provide a mechanically and electrically stable environment for either native or purified channel proteins. However, this approach may not be suitable for all applications. Bilayers produced in this way will contain some solvent which may, in theory, affect channel performance. Although it should be noted that where comparisons of channel activity have been carried out using solvent-containing and solvent-free systems, bilayer solvent does not appear to have significant adverse effects (Labarca *et al.* 1980; Latorre, 1986; Moczydlowski *et al.* 1984).

Both painted bilayers and folded bilayers formed on a partition have the important advantage that the investigator has ready access to, and can easily control, the constituents of the solutions on both sides of the bilayer.

(2) *Resolution.* The usefulness of a particular system of ion channel reconstitution may be limited by the relative size of the inherent background noise of the system and the signal under investigation. It is impractical to paint bilayers on holes with diameters much below 100 μm. The large surface area of such a bilayer in comparison with an equivalent bilayer formed at the end of a patch pipette with a diameter of approximately 1 μm means that the painted bilayer will be considerably more prone to electrical and mechanical noise. It may not be entirely coincidental that the majority of channels investigated in painted bilayers have high single-channel conductance.

Bilayer noise can be reduced by low-pass filtering but this will limit the resolution of channel open and closed lifetimes. Placing the experimental chamber in a metal box will screen the system from electrical interference and mechanical vibration can be reduced by siting the experimental chamber on some form of vibration isolation system (Alvarez, 1986).

(3) *Bilayer electronics.* The principles governing the measurement of single-channel current deflections are the same whether the channel is in its native cell membrane or reconstituted into an artificial bilayer, and the apparatus used for monitoring channels incorporated into bilayers on patch pipettes is identical to that used in conventional patch-clamp experiments. However some differences do arise when channels are incorporated into large planar bilayers. Under these conditions there is essentially no series resistance. The large surface area of the bilayer and hence the much larger capacitance of this system compared with a bilayer on a patch pipette means that capacity compensation is considerably more difficult to achieve.

The circuit layout used in our laboratory for the measurement of single-channel current fluctuations in large planar bilayers is shown in Fig. 6. The bilayer chambers are connected to the circuit via 2% (w/v) agar bridges in 3 M LiCl and silver-silver chloride electrodes (see Fig. 1). High ionic strength salt bridges are used to minimize liquid junction potentials for measurements under mixed ion conditions. The reed switch located between the input sockets connecting the silver-silver chloride electrodes to the circuit can be activated with a magnet. When activated, the reed switch shorts out the chambers and prevents the development of large voltages across the bilayer during chamber perfusion or additions to the chambers.

The requirements for the current to voltage converter A1 (OPA102BM from Burr-Brown, 1 Millfield House, Woodshots Meadow, Watford, Hertfordshire,WD1 8YX, UK), are: (a) low input bias current, (b) low noise, (c) high speed.

Alas these are to some extent mutually exclusive, resulting in some manufacturers using discrete components to fabricate head amplifiers. For simplicity's sake the use of a low noise electrometer operational amplifier together with a frequency compensation circuit yields a head amplifier with reproducible characteristics, simple construction and easy setting up.

R1 is a critical component, needing to be at least 1% tolerance, to ensure an

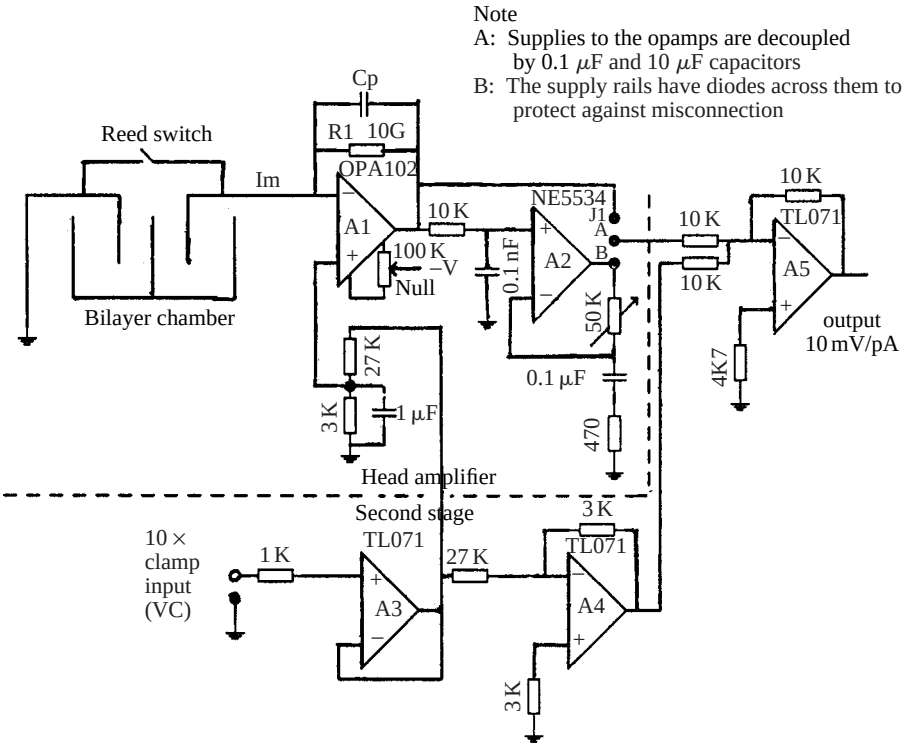

Fig. 6. The bilayer chamber consists of *cis* and *trans* chambers (Fig. 1). The *trans* chamber is held at virtual ground whilst the *cis* chamber may be clamped at a potential relative to ground. The bilayer current is converted to a voltage by amplifier A1 and resistor R1, to give an output of V=–(Im × R1) + (VC/10). Amplifier A2 applies frequency compensation. The output of this stage is passed to the second stage where it is summed with a voltage equivalent to - (VC/10), to give an output Im × R1.

accurate output, and needs to have a good 'lumped' parasitic capacitance. We have found resistors from KOBRA (123 Interstate Drive, W. Springfield, MA 01089, USA) to be satisfactory. The parasitic capacitance associated with R1 causes the output to be limited in rise time, as the capacitance has to be charged through R1. With R1=10 GΩ this capacitance gives a rise time of several ms! The frequency compensation circuit corrects this to approximately 200 μs. With smaller values of R1 the parasitic capacitance has less effect, allowing faster rise times, with concomitant lower output (1GΩ=1 mV/pA etc). The frequency compensation circuit is effectively a frequency sensitive amplifier, amplifying fast changing signals more than slow changing ones, and falling to unity gain at DC. This means that it will preferentially amplify any noise from the bilayer. R2 and C1 act as a low pass single pole filter to cut out high frequency noise before frequency compensation. The frequency compensated signal will still be more noisy than the non-frequency

compensated (there is no such thing as a free lunch!). For noise reasons the command voltage to the head amplifier is supplied at 10× the required value and reduced to the required value at the head amplifier. This also reduces any noise impressed on the signal. The signal is also filtered by a capacitor across the 3 K resistor. This also limits the rise time of any command signal and therefore may be undesirable. As with the parasitic capacitance of R1 above, the membrane capacitance has to be charged through R1. This results in amplifier saturation on the application of a step change in VC. Due to the large capacitance associated with bilayers, around 47 pF for a 200 μm hole, as opposed to patch pipettes, capacity compensation for the membrane is difficult to implement in the usual fashion due to the limited voltage excursion of operational amplifiers.

The output of the head amplifier is a voltage equivalent to $-(Im \times R1) + (VC/10)$. To remove the contribution due to VC the second stage sums the head amplifier output with an inverted signal equivalent to VC/10.

The second stage supplies power to the head amplifier. This should be a low noise supply. A stirrer supply is also needed and can be run from the second stage. It is important to ensure that both leads to the stirrer are grounded when the stirrer is switched off. Star earthing should be used throughout to minimise earth related noise problems.

9. Conclusion

Ion channel reconstitution is an invaluable technique for the investigation of the properties of channels from intracellular membrane systems such as the endoplasmic or sarcoplasmic reticulum. It also provides an essential assay system for the characterisation of the properties of purified channel proteins. I hope that this chapter provides a useful introduction to the diversity and flexibility of the various approaches available to the potential ion channel "reconstituter".

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