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Reviews

Patch clamp technique and biophysical study of membrane channels

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Summary. The present work describes the patch clamp technique, which first allowed the recording of single channel currents in biological membranes. In particular, it describes procedures for preparation and applications of the four different patch clamp configurations. Briefly, the cell-attached configuration is widely used for investigating channel modulation by transmitters acting via second messengers. The cell-free configurations (inside-out and outside-out), complementary to one another with respect to the orientation of the membrane surface, are particularly indicated for the study of the biophysics (kinetics, conductivity, selectivity, mechanism of permeation and block) of ionic channels. Finally, the whole-cell configuration which, because of the remarkable feature that it allows voltage clamp of very small cells, has given access to a number of physiologically important preparations never studied before.
Key words. Ionic channels; patch clamp; single channel recording.

Introduction

The physiological basis of cell excitability is the voltage-dependent and ion-selective permeability of the cell membrane[1,28,29,36,55]. Experimental evidence indicates that the two properties do not apply to the entire membrane surface, but are restricted to specific sites known as channels. These are proteic structures spanning the entire lipid bilayer, and randomly distributed within it. They serve the purpose of reducing markedly the otherwise high energy barrier of the lipid matrix to the movement of ions. For a long time the presence of different types of channels and their properties, notably the all-or-nothing behavior of their opening and closing kinetics, and their independent activity, have been inferred from macroscopic measurements. Conductance relaxation and current fluctuation ('noise') analysis have been the most popular techniques for gaining information about gating kinetics of the channels and their conductance, whereas selective blockers and drugs, and solution replacement, have been extensively used to characterize different channels[27].

Since the recent advent of the patch clamp technique[12,25,51,57,58] the ionic channels can be studied individually, and their properties, such as conductance, selectivity, voltage dependence, and activation by agonists, assessed directly. More relevant information can also be obtained about the opening and closing kinetics, and the mechanisms governing these processes. Not only has the patch clamp technique opened a new perspective on the biophysical basis of ionic channels, it has also given access to a number of physiologically and medically important preparations which, because of their small size, had resisted previous electrophysiological approaches. These are red blood cells[21,23], chromaffin cells[17,18], sinoatrial node cells[59], pancreatic beta cells[47], rat clonal pituitary cells[22], *Limulus* photoreceptors[3], human macrophages[20], vestibular hair cells of the chick[53], acinar cells of lacrymal gland[19], human T lymphocytes[15] and isolated rods of the salamander retina[2]. The aim of this review is to describe succinctly the essential features of the patch clamp technique, and to illustrate the various ways it can be used.

The patch clamp technique

General

In order to resolve electrophysiologically elementary currents through single ionic channels of biological membranes, two requirements have to be met. Firstly, the number of channels under study has to be moderate so that there are times during which only one or at most a few of them are active. Secondly, the background noise during the measurement of these very small currents of only few picoampere (pA = 10^{-12} A) has to be low. The patch clamp technique largely meets both requirements. In particular, the first is met by placing suitable micropipettes (0.5–1.0 μm tip diameter) onto the surface of the cell in order to isolate electrically a part of the membrane containing one or only few channels. The second is met by carefully designing the electronic circuitry to keep instrumentation noise sufficiently small. This was how the first single channel recording was obtained[51]. The original arrangement suffered, however, from two major restrictions due to the imperfect (< 20 MΩ) seal between the pipette and the bath solutions (fig. 1): 1) The potential

inside the pipette had to be held near the bath potential in order to prevent a large and noisy leakage current from flowing through the seal conductance; 2) current traversing the patch membrane partially escaped recording through the pipette because of the shunt through the seal conductance.

Both limitations were virtually removed a few years later when it was noticed that a tight seal (henceforth called gigaseal because of its gigaohm (10^9 Ω) resistance range) between the glass pipette and the cell membrane could be obtained by applying negative pressure through the pipette[25,50,62]. This maneuver can be successful only if great care is taken to ensure cleanliness of the membrane surface, patch electrode tip, electrode and bath solutions. Generally, the membrane surface of cells grown in culture is sufficiently clean to allow satisfactory seals. In contrast, cells from adult tissues always require some enzymatic treatment, both to isolate the cell from the intact organ, and to remove remnants of connective tissue from the membrane[3,35,40,50,53]. The endowment of the patch clamp technique with the high resistance (10–100 gigaohm) gigaseal was undoubtly a significant improvement. Far better resolution of the elementary currents through the patch membrane could be obtained, and the fraction of current shunted by the seal conductance became negligible. More conspicuously, the potential in the pipette could now be varied with respect to the bath potential, so the full range of voltage-clamp experiments became possible. Elimination of the need to clamp intracellular voltage through two microelectrodes, as was still necessary in the original work of Neher and Sakmann[51], has simplified experimental work, and created a better chance to approach very small cells, as mentioned in the previous section.

Formation of a gigaseal

As already mentioned, when a fire-polished pipette comes into contact with a cell membrane, a glass-membrane seal of a few tens of MΩ is established. This resistance can however be raised to the gigaohm range by applying gentle suction (of the order of 10–20 ml of water) through the patch pipette. This action leads to physical distortion of the cell membrane which is partially sucked into the pipette (fig. 1). An estimate of the extent of the cellular membrane area invaginated by this process was given by Sakmann and Neher[56]. They reported that for a pipette with 2.5 MΩ tip resistance, the membrane area invaginated in the omega-shaped figure was 14 μm^2. The authors also gave evidence that half of this membrane area was tightly engaged in the glass-membrane seal, whereas the remainder was in free contact with the pipette solution (fig. 1). It is very likely that the increased contact area between the inner wall of the pipette and the cell membrane favors the formation of the gigaseal. In order to explain in physical terms the type of interaction occurring between the glass and the membrane, the following three forces have been considered as the major determinants of the sealing process[13]: 1) salt bridges involving divalent cations such as calcium; 2) hydrogen bonds; and 3) van der Waal's forces in the final stage of the process, when glass and membrane surfaces are in close contact. The distance between the two surfaces has been estimated to be no larger than 2–5 Å[12], as necessary to produce the resistance of 50–200 GΩ. It remains unknown whether this figure represents a true limit, or whether better seals can be obtained. The highest seal resistance reported so far amounts to 400 GΩ[2]. More often, however, seal resistances are in the range of 10–50 GΩ. These seals are nevertheless well suited for resolving elementary ionic currents through most membrane channels. In addition to the electrical characteristics just described, gigaseals display an unexpected physical strength. They can be widely manipulated without major deterioration of the glass-membrane seal, as we shall see in the following sections.

The four configurations of the patch clamp technique

Cell-attached configuration

This is the initial configuration after establishing a gigaseal (fig. 1). The cell-attached configuration is thus the easiest to obtain. It also disturbs the membrane and its

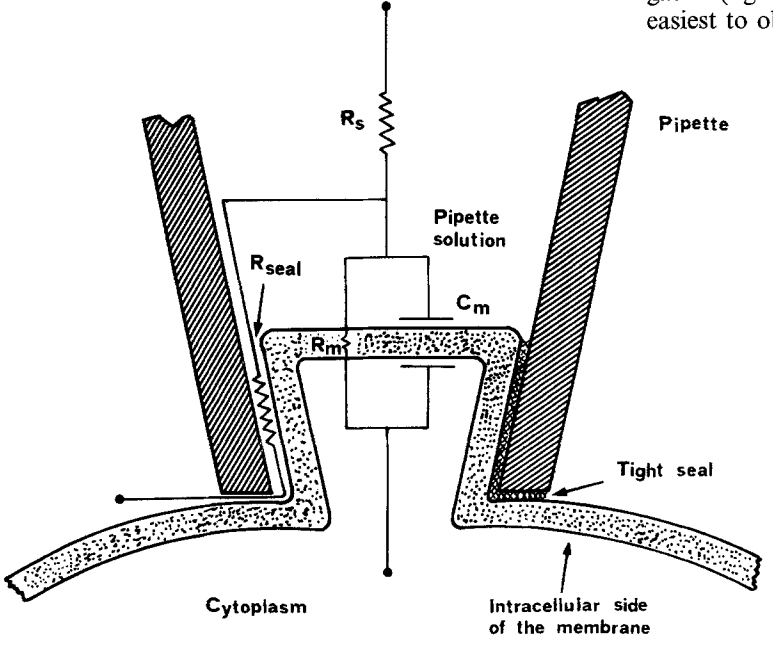

Figure 1. Schematic drawing of the pipette-membrane arrangement after the gigaseal formation, as well as its equivalent circuit (see text for discussion).

physiological environment to a lesser extent than other configurations. Current of both voltage- and transmitter-activated channels can normally be studied by either varying the membrane voltage, or using pipettes filled with solutions containing the transmitter[12,14,33,34,43,51,54,62]. Nevertheless, some desirable experimental manipulations are not possible with this configuration. Neither the extracellular nor the intracellular solution can be changed. This means that experiments concerning dose-response characteristics of drugs and transmitters or channel ionic selectivity cannot be explored for a particular patch. A statistical approach, with the inevitable larger number of measurements, is necessary. Attempts have been made to change the pipette solution[14], but this appears to be a difficult and troublesome remedy.

The cell-attached configuration has one prominent feature. Since a seal resistance greater than 10 GΩ is consistent with glass-membrane separation such as to prevent the diffusion through it of even small molecules, this configuration separates the whole cell membrane into two parts: the few square microns of membrane surface under the patch pipette (the 'free area') in contact with the pipette solution, and the rest of the membrane dipped in the bath solution. (This separation has been acknowledged by the evidence that the application to the bath of ACh at concentrations as high as 10 μM does not activate single channels in the patch area covered by the pipette, proving that ACh diffusion through the seal is negligible.) This unique feature of the cell-attached configuration can be exploited to perform meaningful experiments; in particular, the involvement of second messengers in channel modulation can be tested. One example is the modulation of K channels by serotonin in *Aplysia* sensory neurones. There was evidence that one of the steps leading to slow changes in K conductance involved protein phosphorylation by a cyclic AMP-dependent protein kinase. The sequence of events proposed by Kuo and Greengard[40] began with transmitter activation of adenylate cyclase, which, through cyclic AMP, activates the protein kinase. Although subsequent results were consistent with the mechanism proposed, direct evidence of such an interaction between spatially separated receptors and channels via a second messenger has only recently been provided. Using the cell-attached configuration, Siegelbaum and coworkers[60] showed that the addition of serotonin to the bath solution consistently decreased channel activity in the patch. A similar decrease in the activity was also obtained by the same authors by intracellular injection of cyclic AMP. These findings are consistent with the notion that these K channels are spatially separated from the extracellular serotonin receptors and that their modulation by serotonin is mediated by an intracellular messenger acting at a distance. Moreover, they are a clear illustration of the suitability of the cell-attached configuration for this type of investigation. Other reports of channel activation upon application of agonists to the bath solution have been published: for ACh and cholecystokinin on glandular cells[49], serotonin on *Aplysia* neurons[60], cAMP and adrenaline on heart cells[7,8].

Inside-out configuration

Because of the high mechanical stability of the glass-membrane seal, further manipulation is possible from the stage of the cell-attached configuration. If the cell under study is mechanically attached to a substrate, quick withdrawl of the pipette from the cell will tear off the patch membrane from the cell, while the glass-membrane seal is maintained. The cytoplasmic (inside) face of the membrane will then be exposed to the bath solution. This configuration is referred to as 'inside-out'. The cell from which the membrane patch has been torn off, often reseals, so more than one patch may be obtained from it. This way of obtaining the inside-out configuration may appear simple in comparison to a number of more laborious procedures described in the literature[25,26,50]. It works, however, routinely with cultured neurons from embryonic rat brain and spinal cord, and myoballs (fig. 2A). It has also been successfully used by other investigators[5,31,64].

Once the cytoplasmic side of the membrane is exposed to the bath solution, it can be easily and repeatedly exposed to a variety of solutions. This configuration has been extensively used to study the dependence on intracellular calcium of a number of Ca^{2+}-activated channels[11,20,45,46,48,63,65], as well as the mechanism of action of agents blocking channel conductance from the cytoplasmic side[4,34,66]. The determination of channel selectivity is another application[6]. With regard to channel modulation by transmitters or hormones, the second messenger hypothesis has been further tested by applying the catalytic subunit of cyclic AMP-dependent protein kinase directly to the cytoplasmic side of the membrane[16].

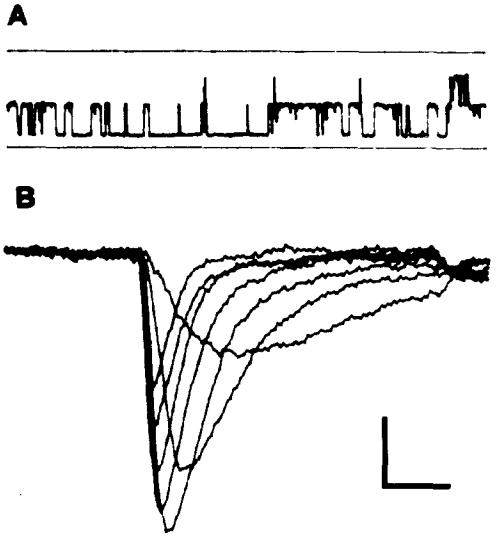

Figure 2. Representative records of single-channel and whole-cell currents. Outward currents are shown as upward deflections. The preparation was a primary cell culture from the hippocampal region of embryonic rat brain. (A) Inside-out configuration. Pipette solution was standard rat Ringer. The record shows currents through two Ca^{2+}-activated K channels. The solution bathing the cytoplasmic side of the membrane was 140 mM KCl. Ca^{2+} concentration was 2×10^{-6} M. Holding potential was 0 mV. Calibration: Horizontal, 250 msec; vertical, 12 pA. (B) Whole-cell configuration. Pipette solution was 140 mM CsCl, 0 mM Ca. Family of records taken over the voltage range + 20 and + 80 mV. Holding potential was − 70 mV. Only sodium currents are present since the content of the pipette, which readily diffuses into the cell interior, is made up of ions impermeant through K channels. Each record is the average of five records taken with the P/4 procedure, to correct for capacity and linear leakage current components. The P/4 procedure consists in applying an ensemble of a test pulse, and four additional pulses of 1/4-amplitude and opposite sign, which are subsequently digitally subtracted. Calibration: Horizontal, 2 msec; vertical, 4 nA.

Outside-out configuration

Comparable to the inside-out configuration, as far as the current resolution and the control over one bathing solution are concerned, the outside-out configuration differs from its counterpart for having the extracellular face of the membrane exposed to the bath solution. The procedure for obtaining the outside-out configuration is, however, more elaborate and usually less successful. It starts from the cell-attached configuration. The next step is the rupture of the patch membrane underneath the pipette. This can be accomplished by applying suction to the pipette solution after the gigaseal has been formed; suction of 50–100 ml of water over 2–20 s is usually required. An alternative approach, perhaps more elegant than effective, consists in applying a strong voltage pulse (up to 300 mV, for 200–500 ms). The rupture of the membrane can be recognized by the increase of the capacitive current in response to a small voltage pulse. The increase is due to the fact that now the entire cell membrane contributes to the capacitive current. For cells large enough to have an input resistance detectably lower than the seal resistance, the rupture is also signalled by an increase of the steady state current flowing into the pipette and recording circuit during the voltage pulse. After the rupture of the patch membrane, the pipette solution communicates with the cytoplasm. When the pipette is now slowly retracted from the cell surface, a cytoplasmic protuberance surrounded by membrane forms initially and continues to maintain electrical coupling. Upon further withdrawal, the bridge thins until it is interrupted. Usually, the membrane attached to the pipette reseals, yielding a patch with its external side in contact with the bath solution. This orientation is particularly suitable for studying the action of transmitter substances and other types of chemicals or ions on the extracellular side of the membrane[10,24,32,61]. Although the outside-out configuration is a valuable experimental tool, its use is more restricted than that of its counterpart. Apart from the more laborious procedure for obtaining it, and a lower rate of success, the outside-out configuration appears to be a more delicate, less stable structure. The reason may be that, contrary to the inside-out configuration in which the patch membrane formed upon withdrawl of the pipette from the cell is securely sheltered inside the pipette, in the outside-out configuration it probably remains more exposed. The flowing of a perfusion bath solution is often sufficient to destroy the membrane or severely affect the seal.

Whole-cell configuration

Whole-cell configuration results from the rupture of the patch membrane at the stage of the cell-attached configuration. Whole-cell configuration provides a low resistance pathway between the pipette solution and the cell interior. This feature was originally used to record macroscopic currents from the entire cell membrane[9,17,22,52]. When whole-cell configuration is established on small cells (cell diameter < 10 µm) whose input resistances are very large compared to the access resistance of the patch electrode, good voltage clamp and reliable current measurement can be obtained (fig. 2b). Unfortunately, this situation rapidly changes as the size of cells increases, and their input resistance approaches values comparable to the access resistance of the electrode. For this reason, the quality of the voltage clamping should be checked whenever larger cells are used.

The whole-cell configuration also represents an effective dialysis technique comparable to those applied to larger cells[37–39,42]. After the rupture of the patch membrane and the establishment of the whole-cell configuration, diffusion tends to equilibrate ions and substances of small molecular weight and, although at a fairly slow rate, larger compounds such as cytoplasmic proteins and even cellular organelles, between the pipette solution and the cell interior, and vice-versa. The time courses of the equilibration for each species are described by exponential functions whose time constants depend on species size. Fenwick et al.[17] report a time constant of 5 s for the exchange of Na between the pipette (150 mM) and the cell interior. Marty and Neher[47] calculated a time constant of about 3 min for a globular protein with a 50-Å Stokes radius, and 30 min for a vesicle 0.1 µm in diameter. Thus, besides a good voltage clamp and current recording from very small cells, this configuration also offers good control of the intracellular solution. As is the case for the cell-attached configuration, the control is normally limited to one choice of the filling solution of the pipette.

As illustrated, the whole-cell configuration represents an intermediate step of the procedure leading to the outside-out configuration. In addition, after the complete withdrawal of the pipette from the cell to obtain the outside-out configuration, the membrane reseals leaving the cell essentially intact, except for the internal solution that has equilibrated with pipette solution. These features suggest a considerable number of interesting experiments. Notably, repetitive measurements in either a whole-cell or outside-out configuration can be performed on the same cell, with the additional chance of being able to change the intracellular solution each time a whole-cell configuration is achieved. Furthermore, both macroscopic and elementary current can be measured on the same cell; this allows the necessary comparison between the two sets of data.

Conclusions

The present survey of the patch clamp technique has mainly focussed on the features and applications of the four different patch clamp configurations. In summary, the cell-attached configuration is especially appropriate for investigating channel modulation by transmitters acting via second messengers. The cell-free inside-out and outside-out configurations, complementary to one another with respect to the orientations of the membrane surfaces, have similar resolutions and possibilities for controlling adjacent solutions. For this reason they are particularly indicated in the study of the biophysical parameters (kinetics, conductivity, selectivity) of ionic channels. The whole-cell configuration, which is costumarily grouped with the patch clamp configurations only on account of its tight seal between cell membrane and pipette wall, has the remarkable feature of allowing voltage clamp of very small cells.

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Full Papers

Effects of mistletoe (*Viscum album* L.) extracts on cultured tumor cells[1]

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Summary. Bacterially fermented mistletoe preparations (BFMP) were tested on rat hepatoma tissue culture (HTC) cells and human leukemia Molt 4 cells. A dose-dependent inhibition of the growth rate of the cells was observed. For both cell lines, cytostatic concentrations, expressed in weight of fresh plant, were 0.5 mg/ml culture medium for oak BFMP and 1 mg/ml for apple tree BFMP. However, the action of the two preparations was markedly different on each cell line. Non-viable HTC cells were not stained by trypan blue while non-viable Molt 4 cells were fully colored by this reagent. A lysis of cellular membranes of HTC cells was observed by electron microscopy. Furthermore, oak BFMP inhibited the growth of virus transformed 3T3-SV40 cells more than that of non-transformed 3T3 cells. In contrast to BFMP, non-fermented extracts and a purified mistletoe lectin showed a greater inhibition of the growth of Molt 4 cells than of HTC cells. Samples withdrawn at different times during fermentation gradually lost their inhibitory effect on the growth of Molt 4 cells while their action on HTC cells increased up to the 4th day of fermentation. These results are discussed in relation to the cytotoxic substances of mistletoe already characterized.
Key words. Mistletoe extract; cytotoxic substances; hepatoma tissue culture; human leukemia cells.

Introduction

Bacterially fermented aqueous extracts of mistletoe have a cytostatic effect on animal tumoral cells in culture[2]. They inhibit the growth of plant roots[3] and plant tumoral tissue in culture[4]. In mice inoculated with tumor cells from different experimental cell lines (Ehrlich ascites carcinoma, Sarcoma 180 and Lewis lung carcinoma) the survival time of treated animals is equal to or higher than that of mice treated with 5-fluoro-uracil, a well known anti-tumoral agent[2]. Moreover, the extracts stimulate humoral and cellular immunity in mice[5,6]. A marked increase of the weight of the thymus, corresponding to a higher proliferation rate of cortical thymocytes, is also observed and this effect is reversible[7].

Several cytostatic or cytotoxic proteins have been characterized in mistletoe, particularly three glycoproteins classified as lectins[8–11] (mol.wt 115 kD, 60 kD and 50 kD), four viscotoxins[12,13], (basic polypeptides of mol.wt around 5 kD) and 10 other basic proteins, the so-called Vester protein complex (VP 16)[14–16] which has potent cancerostatic and immunomodulatory properties. Moreover, the presence of cytotoxic alkaloids[2] and immunomodulatory polysaccharides[17] has been described. The amount of lectins in bacterially fermented preparations is 5–10 times lower than in non-fermented preparations[18]. It is not known whether lectins are metabolized during bacterial fermentation or slowly degraded by storage. Viscotoxins are quite resistant to degradation[19]. The VP 16 complex loses a large part of its biological activity upon storage[16].

In this paper we describe the inhibitory effect of bacterially fermented aqueous extracts of mistletoe on the growth of cultured cells in vitro and their effects on cell morphology and ultrastructure. The results are compared with those obtained with non-fermented extracts of mistletoe and with samples taken at various times during the fermentation process.