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CYTOCENTERING: A Novel Technique Enabling Automated Cell-by-Cell Patch Clamping with the CYTOPATCH™ Chip

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Automats for patch clamping suspended cells in whole-cell configuration must (1) bring isolated cells in contact with patch contacts, (2) form gigaseals, and (3) establish stable intracellular access that allows for high quality recording of ionic currents. Single openings in planar substrates seem to be intriguing simple solutions for these problems, but due to the low rate of formation of whole-cell configurations we discarded this approach. Single openings are not suited for both attracting cells to the opening by suction and forming gigaseals with subsequent membrane rupture. To settle the three tasks with a mechanical microstructure we developed the so-called CYTOCENTERING technique to apply to suspended cells the same operation sequence as in conventional patch clamping. With this method we immobilized selected cells from a flowing suspension on the tip of a patch pipette by suction with a success rate of 97% and formed gigaseals with a success rate of 68%. Subsequent whole-cell recordings and intracellular staining with Lucifer yellow proved the stable access to the cytoplasm. Currently, a chip with an embedded suction opening in glass surrounding the microstructured contact pipette is under development. The processing of this CYTOPATCH™ chip is compatible to large-volume production. The CYTOPATCH™ automat will allow for fully automated, parallel, and asynchronous whole-cell recordings.

Keywords Automated Patch Clamping, Cytocentering Technique, Drug Discovery, High Information Content, High-Throughput Screening, Ion Channel, Secondary Screening

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INTRODUCTION

The knowledge about structure, function, and regulation of ion channels, their involvement in various diseases, and the functional changes that causes channelopathies has strongly increased (Marban 2002; Weinreich and Jentsch 2000). Accompanied by the rising interest in membrane proteins, ion channels receive special attention as a target class for drug discovery by the pharmaceutical industry. To meet the needs of industrialized drug discovery, adequate methods and instrumentations are required for screening a large number of compounds against this target class. Undoubtedly, electrophysiological methods that directly monitor the modulation of specific ion currents as a measure for the pharmacological activity of a compound are preferred.

The Conventional Patch Clamp Technique

The most powerful method for detailed biophysical investigation of function and regulation of ion channels is the patch clamp technique (Hamil et al. 1981). It allows control of the voltage of the host cell under selected ionic concentrations and, hence, monitoring of the gating of ion channels under defined conditions. At the single cell level it enables the coupling of functional and molecular studies on ion channels (Monyer and Lambolez 1995) and gene expression analysis (Dixon et al. 2000).

The method is based on the formation of a very close contact of the cell membrane and the tip of a glass pipette as the result of a series of well-defined steps as shown in Figure 1A. After selecting a cell attached to the bottom of a culture dish, the tip of a patch pipette is moved to the cell and finally pressed slightly

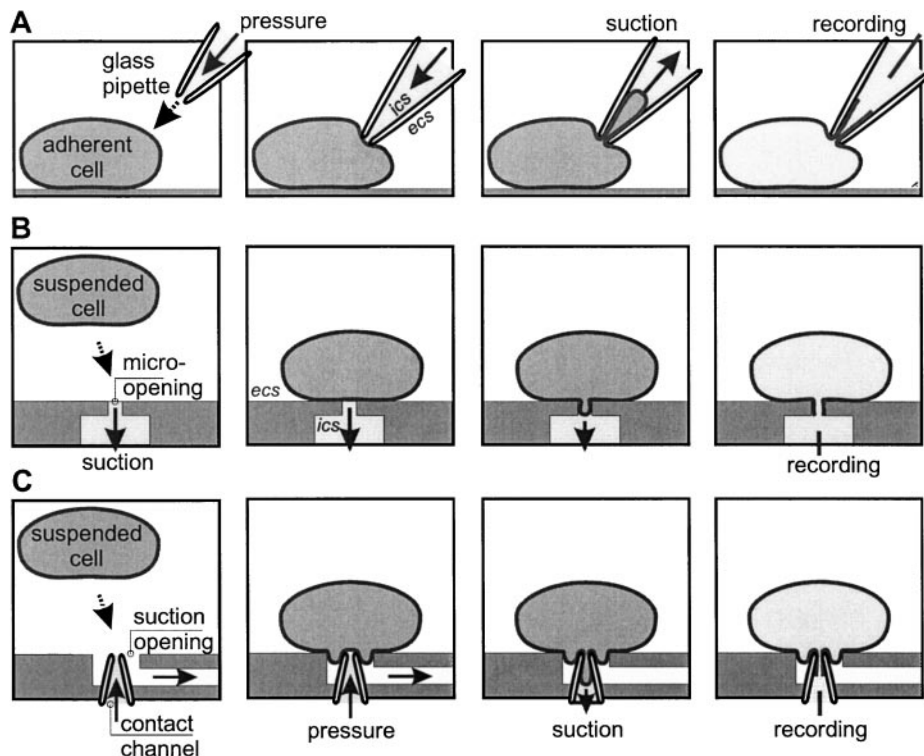

FIG. 1. Patch clamp methods. A) Operation sequence of the conventional patch clamping. The tip of a glass pipette is brought in contact with the membrane of an adherent cell. Finally, after application of a well-defined operation sequence, the membrane patch under the tip of the pipette is ruptured. This enables intracellular access for clamping the membrane voltage and detecting the current flowing through ion channels; *ics* intracellular solution, *ecs* extracellular solution. B) Operation sequence with the planar patch approach. The micro-opening both serves for suction application to attract suspended cells and for sealing and recording. C) The same operation sequence as in the conventional way is applied with the CYTOCENTERING technique. A suspended cell is attracted to the tip of a substrate-embedded contact channel by application of suction to an opening surrounding the contact opening.

against the cell membrane. During this step a positive pressure is applied to the inside of the pipette to avoid dirt sticking to the pipette tip. Under these conditions, the membrane under the pipette becomes imprinted with a concave curvature. Then, a slight negative pressure is applied that results in pulling a part of the membrane into the tip opening. The contact between membrane and glass surface gives rise to a seal formation with an ohmic resistance in the gigaohm range, the so-called gigaseal, measured between pipette solution and electrolyte surrounding the cell and the pipette.

After establishment of a gigaseal, the membrane under the patch pipette is disrupted by suction or short voltage pulses. With this configuration, the ionic currents through the whole-cell membrane can be studied via the low resistance access to the cytoplasm (Hamil et al. 1981). Prerequisites for success rates for gigaseal formation up to 80% are (1) unused patch pipettes with clean tip and inner surface, (2) gentle approaching of the pipette tip to the cell membrane with the patch pipette perpendicular to the membrane plane, and (3) smooth and also clean cell membranes.

The gigaseal provides low background noise of the recording and mechanical stability of the contact (Neher 1992). Despite the widespread use of the patch clamp technique over the last decades, the true nature of the membrane-glass contact and the resulting high resistance seal formation is not understood in detail. It is assumed that it results from a molecular interaction between a patch of membrane pulled into the pipette and the clean and hydrophilic surface of the inner pipette wall at a distance of few Angstroms between membrane and glass surface (Corey and Stevens 1983; Opsahl and Webb 1994; Sakmann and Neher 1983).

Automation of the Patch Clamp Technique

Conventional patch clamping measurements are carried out on cells that are immobilized by adhesion to a substrate. It is a cell-by-cell assay that is slow due to the multiple-stage operation sequence that is manually handled by a well-skilled operator. Opposite to this is the need of today's drug discovery for accelerated ion channel screening. Therefore, at present, strong efforts are undertaken to make this technique applicable for higher throughput screening of ion channel targets and pharmaceutical substances acting on them. The most common approach underlying the developments for automation, miniaturization, and parallel execution of the patch clamp technique is the replacement of the patch pipette by a microstructured planar substrate containing a microopening for mechanical and electrical cell contacting (reviews about the automation approaches by Owen and Silverthorne 2002; Xu et al. 2001; Sigworth and Klemic

2002). This approach is based on the idea to reverse the conventional method: Note the pipette is brought to immobilized cells but suspended cells to a fixed contact opening. This requires attracting cells to a certain position as, for example, by suction (Fertig et al. 2002; Mathes et al. 2001) or electrical fields (Guia et al. 2002; Schmidt et al. 2000), and contacting them with a microstructure suitable for seal formation and for enabling a stable intracellular access.

The Planar Patch Approach

The easiest arrangement for the replacement of the patch pipette is a simple opening in a planar substrate used both for cell attracting by suction and cell contacting as shown in Figure 1B. With this arrangement, sealing can occur by cell adhesion in the planar contact area surrounding the opening and along the wall of the channel penetrating the substrate when the membrane is pulled into the opening. A similar technique has been described first by Kostyuk, who used channel-like pores in polyethylene films in which isolated neurons were fixed by suction for intracellular perfusion and voltage clamping experiments (Kostyuk et al. 1975).

This initial planar patch approach was the focus of a research project at the NMI Natural and Medical Sciences Institute over a period of several years. We employed single microopenings (diameter 1 to 4 μm) in thin polyimide films (thickness 7 μm) to attract and contact primary cells (sheep cardiac Purkinje cells), but failed to yield an acceptable success rate for gigaseal formation and whole-cell recording (Stett, Bucher et al. 2002). We learned that adhesion in the planar contact area, which surrounds the openings, is not sufficient for tight contacts (Stett, Knott et al. 2002). In accordance with the state of knowledge and experiences of other groups (Sigworth and Klemic 2002) it became clear that, for reliable sealing, elongated channels in substrates with a thickness of more than 10 μm and an opening diameter smaller than 2 μm are preferable (Fertig et al. 2002; Klemic, Klemic et al. 2002; Mathes et al. 2001; Xu et al. 2002).

In the following we present the proof of principle of a new approach that combines the advantage of openings with diameter larger than 4 μm for cell attraction by moderate suction and openings smaller than 2 μm on channels for seal formation.

We then describe the current state of the realization of a corresponding patch clamp chip, the CYTOPATCH™ chip. This chip will replace the patch pipette and all means for cell positioning. It will allow to apply the same protocols for whole-cell analysis as the conventional whole-cell patch clamping does—with the same quality and information content.

RESULTS

The Cytocentering Approach for High Quality Patch Clamping

In view of the low yield of successful recordings with single opening approaches, (Fertig et al. 2002; Klemic, Klemic et al. 2002; Owen and Silverthorne 2002; Sigworth and Klemic 2002) we reconsidered the features crucial for success in the conventional patch clamp procedure as described above. We identified the procedure for positioning of cells, the cleanness and smoothness of surface of the contact channel, and the diameter of the contact opening as more important than geometry and material of the channel.

In a pragmatic approach we then mapped the conventional patch clamp procedure in an inverse configuration. The principle is to move suspended cells by a fluid flow to a suction opening that is used to bring a membrane patch in contact with a micromachined patch contact centered inside the opening as shown in Figure 1C. After cell positioning, the patch contact is used for patch clamping as in the conventional way. This approach enables the application of an operation sequence with the same hydrodynamical and mechanical forces acting on the cell and its membrane as in the conventional patch approach since the placement of the cell on the contact tip also causes an imprinting with a concave curvature of the membrane under the tip.

Proof of Principle

To prove that cells can be selected from a flowing suspension and positioned by suction on the tip of a patch pipette without damaging the cells we used a setup as sketched in Figure 2. With micromanipulators, a conventional patch pipette was vertically positioned with the tip upwardly placed in the center of a singular opening in a polyimide sheet. This arrangement allowed applying negative pressure to the patch pipette and the surrounding suction opening, as well as performing voltage and current

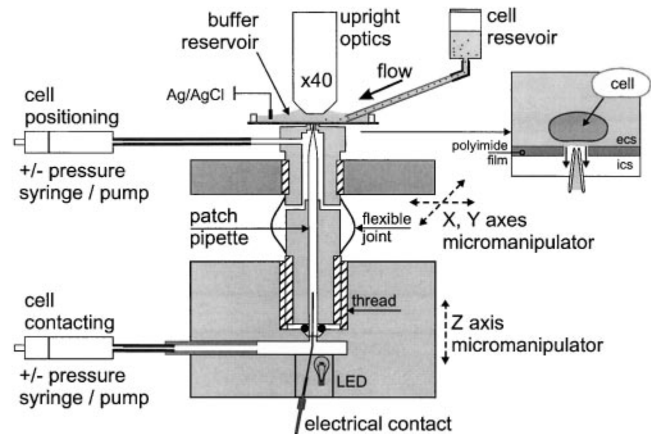

FIG. 2. Assembly for proofing the cytocentering principle. The polyimide film is span over an opening in a DELRIN™ (DuPont) cylinder and fixed with glue. This assembly is mounted to a micromanipulator with X,Y-drive. A second cylinder that is mounted to a micromanipulator with a Z-drive contains a patch pipette with the tip positioned with the manipulator and fixed into the opening in the polyimide film (see insert upper right). Inlets allow the application of positive and negative pressure both to the pipette and to the lower side of the opening in the polyimide sheet. A red LED is located under the lower end of the patch pipette to feed light into the pipette for illuminating the tip of the patch pipette. For cell supply, a cannula connected with a cell reservoir is placed by means of a micromanipulator with X,Y,Z-drive in the buffer reservoir.

clamp measurements on immobilized cells under visual control with an upright microscope. A laminar flow of cell suspension was generated by means of a cannula that was connected with a cell reservoir and positioned in the buffer reservoir with its tip pointing toward the suction opening.

Cell Positioning

Neuro2A cells were cultivated and brought in suspension as described below. With a setup sketched in Figure 2, cells were moved to the contact site under visual control by a gravity driven continuous fluid flow. To prevent uncontrolled attachment of cells to the contact site and flowing of extracellular solution into the patch pipette positive pressure was applied both to the suction and contact opening. When a proper cell reached the vicinity of the contact site negative pressure was applied to the suction opening in order to attract the selected cell toward the openings. We found a diameter of the suction opening in the range between 8 and 12 μm as suitable for reliably attracting cells from a distance of up to 60 μm by application of negative pressure up to 10 mbar to the lower side of the opening. When leading cells into close vicinity of the opening by the flow only slightly negative pressure (about 1 mbar) for further attracting a certain cell to contact opening was required.

With these manipulations, cells were immobilized on the tip of the patch pipette in a well-centered fashion as shown in Figure 3. Thereafter, the suction to the opening was released. Due to the centered attachment, this kind of bringing a cell in contact with the tip of a patch pipette was called the CYTOCENTERING technique. After further improvement of the technique, the success rate of cytocentering Neuro2A cells finally was 97% (37/38 positioned cells). The success criterion was the controlled and centered immobilization of the cell and a distinct increase of the resistance measured between the pipette solution and the reference electrode.

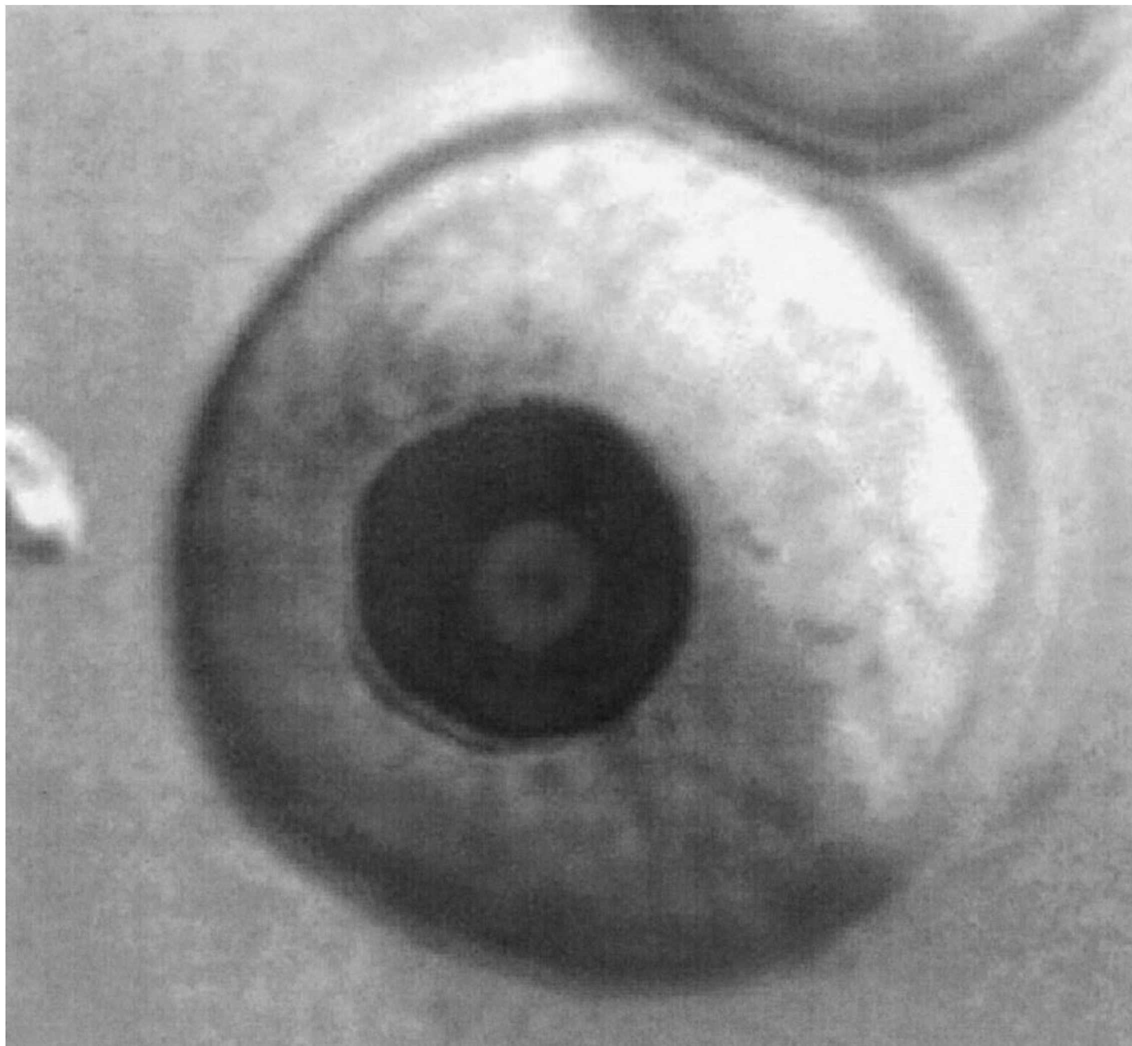

FIG. 3. Cytocentered Chinese hamster ovary (CHO) cell placed on a suction opening with 12 μm diameter (dark circle). The illuminated tip of the patch pipette positioned in the center of the opening also can be seen.

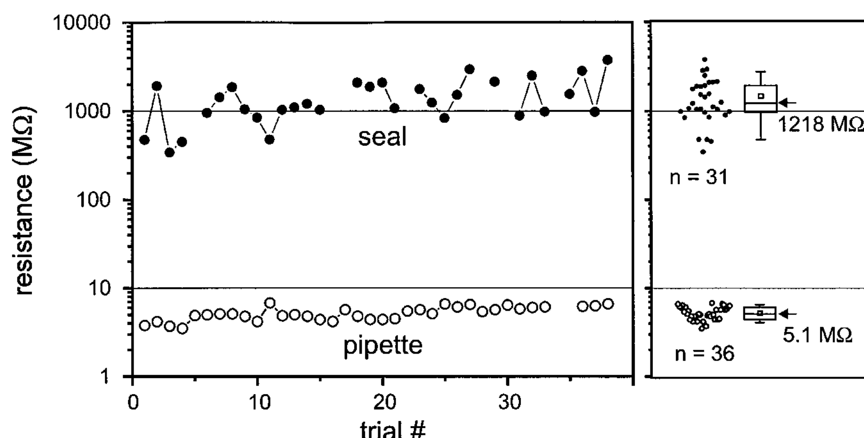

FIG. 4. Experimental seal resistances with Neuro2A cells. A) Individual results from subsequent trials showing the pipette resistance without cell (open circles) and the final series resistance (filled circles). B) Box chart showing the first, second (= median value, arrow), and third quartile (horizontal lines) of the results.

Seal Formation and Whole-Cell Configuration

In the course of the experiment, we continuously measured the resistance between the pipette solution and the reference electrode placed in the buffer reservoir. When a cell was cytocentered, positive pressure to the pipette was released resulting in an increase of the seal resistance. With a slight negative pressure applied to the pipette the final seal resistance was obtained. The results from subsequent runs with Neuro2A cells after initial improvements are shown in Figure 4. The median value for seal resistance was 1.2 GΩ (n = 31) with 68% of the resistances above 1 GΩ (n = 21/31, 6/37 cells formed instantaneously whole-cell configuration).

With the final settings of experimental parameters and the final procedure for cell treatment we unambiguously could prove a whole-cell configuration in 77% (n = 7/9) by measurements of voltage-gated sodium and potassium currents as shown by the typical recordings in Figure 5A and 5B. The data obtained from the suspended cells are in good agreement with data obtained from adherent cells (data not shown). In addition, intracellular staining with Lucifer yellow as shown in Figure 5C was successful in two of five experiments. This is comparable to success rates in conventional patch-clamp experiments.

Development of the CYTOPATCH™ Chip

Using patch pipettes fabricated by pulling glass capillaries is not well suited for full automation and high throughput screening. With the same entitlement to signal quality and information content as in the conventional way each contact has to be a single-use contact to guarantee reliable gigaseal formation. These demands can be fullfilled by the use of substrates with microstructured patch contacts as our envisioned CYTOPATCH™ chip. Figure 6A shows a design pattern of the

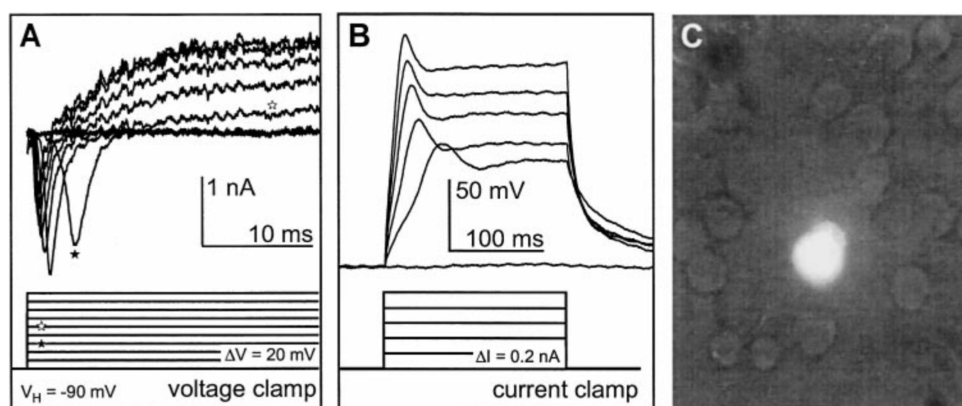

FIG. 5. Intracellular access to cytocentered Neuro2A cells. A) Sodium and potassium currents elicited with activating voltage steps in the voltage-clamp mode (holding potential −90 mV, voltage steps with increment 20 mV from −90 to +90 mV as indicated by the lower voltage traces). Seal resistance 2.5 GΩ. Leakage and capacitive currents have been cancelled out by subtraction of a scaled subthreshold trace, no series resistance compensation was applied. Note the stars indicating threshold for sodium (filled star) and potassium current (open star), respectively. B) Membrane potential measured in the current clamp-mode (holding potential −60 mV, current impulses 0.2, 0.4, . . . 1 nA as indicated by the lower current traces). Seal resistance was 0.9 GΩ. C) Fluorescence image after cytocentering a cell and subsequent intracellular Lucifer yellow staining.

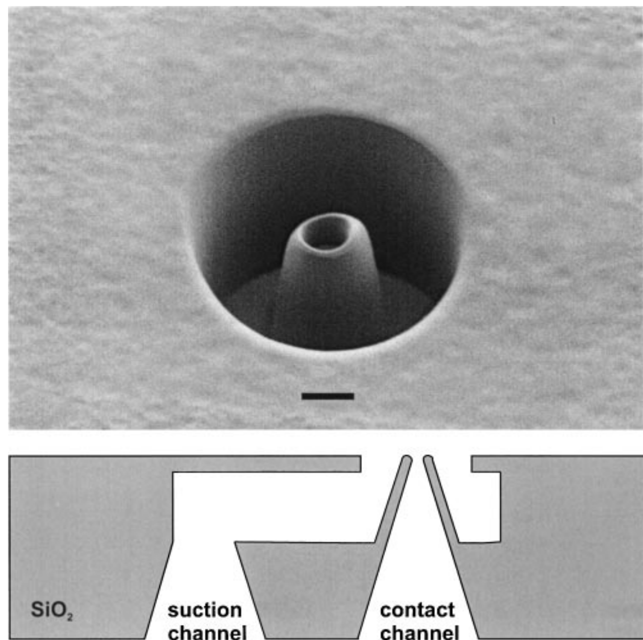

FIG. 6. Prototype of the CYTOPATCH™ Chip. A) Design pattern of the cytocentering site with its two concentric openings formed with a focused ion beam in a 10 μm thick quartz layer (SEM image, scale bar 2 μm). B) Schematic drawing of the cytocentering site with suction and contact channels embedded in the bulk of a quartz chip.

CYTOCENTERING site with the tip opening of the contact channel and the surrounding suction opening. This early prototype microstructure has been formed with a focused ion beam in quartz glass (silicon dioxide, SiO_2) that offers several advantages. Compared to other glasses proofed as materials for making suitable patch pipettes as, for example, borosilicate, sodium or alumina glasses, quartz glass has outstanding electrical characteristics. Since no other compounds other than SiO_2 are incorporated, it is free of movable charges that result in a low dielectric constant (3.7). Together with its high resistivity (10^{14} Ω cm) this endows quartz glass with low noise and excellent insulation characteristics (Corey and Stevens 1983; Levis and Rae 1993). Due to its widespread use in semiconductor and microsystems industries there are well-established techniques for three-dimensional structuring of this material. The manufacturing of this chip has to be compatible to large-volume production. Therefore, we are designing and developing a patch-clamp chip as sketched in Figure 6B that can be manufactured with standard processing steps in a specialized microsystem foundry (Onstream MST, Eindhoven, The Netherlands).

Requirements for an Automat for High Quality Patch Clamping

The CYTOPATCH™ chip is designed to provide the means for cell positioning and to replace the patch pipette for high quality whole-cell recording with a high success rate. It will be a disposable that is reloaded automatically for each cell into the CYTOPATCH™ automat. Since we focus on the quality of the individual recordings prior to throughput, this automat will be first delivered as a single cycle device for cell-by-cell recording. Designed for unsupervised and overnight operation, the chips, compounds, and cells will be supplied to the CYTOPATCH™ site fully automatic (Figure 7). The CYTOPATCH™ automat will enable the same protocols for whole-cell applications with the same quality and information content as the conventional whole-cell patch clamping does. Part of this concept is the integration of interfaces to drug delivery systems that are used in academic as well as in industrial laboratories.

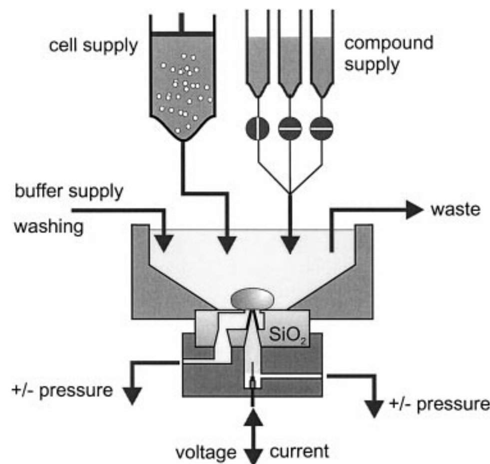

FIG. 7. Schematic drawing of a CYTOPATCH™ site, which provides chip, cell and compound supply fully automated for cell-by-cell patch clamping.

Depending on the type of the performed patch clamp protocol the estimated throughput with one CYTOPATCH™ site will be in the range of up to 200 cells per day. For higher throughput the automat can be equipped with multiple CYTOPATCH™ sites. Each of the sites will be handled individually for asynchronous recording of parallel-patched cells.

DISCUSSION

As it is expected that ion channels will become the major target class for drug screening on nervous system, cardiovascular, and other main diseases, appropriate automated electrophysiological screening methods are required. One of the mainstream technologies for ion channel assays in the basic research, secondary screening, and safety screening market is the automated patch clamping. In this field, sensitivity, selectivity, and high information content of an experiment are more valued than high throughput (Xu et al. 2001).

High quality, automated patch clamping can also be instrumental for the growing biotechnology market for applications in functional genomics, where scientists examine the function of the more than 30,000 recently uncovered human genes by incorporating these genes into strains of cell cultures. Automated patch clamping will help to pick out cell culture strains that incorporated certain ion channel genes (expression cloning) or screen all strains for all types of ion channels (expression screening).

We propose a patch clamp automat based on the new CYTOCENTERING technique for cell positioning and contacting. It solves the problems of planar patch electrodes by structural separation of the cell positioning and cell contacting tasks by means of two concentric openings in a planar chip. No further means for cell positioning like microstructured (Xu et al. 2002) or macroscopic electrodes (Schmidt et al. 2000) for application of electric fields are necessary. Also, it is not necessary to use dense suspensions of cells to improve the chance of a single cell to attach to the recording site (Klemic, Li et al. 2002). The proven CYTOCENTERING principle enables high yield positioning and gigaseal formation by application of an operation sequence that is applied by all electrophysiologists carrying out patch clamping since its introduction to the scientific community. The high yield of positioning and gigaseal formation is permitted by the reliable centering of the cell on the tip of the patch contact. Similarly, in the conventional way, perpendicular attaching of the cell membrane with the patch pipette is one of the precautions that is necessary for high yield seal formation.

The philosophy behind the CYTOPATCH™ automat is to retain the quality and flexibility of the conventional patch clamping prior to escalating the number of data points performed in unitary time. We abstain from the challenge of developing a parallel patch clamp system for high throughput assays based on a two-dimensional array of recording sites, e.g., in the 96-well plate format. Despite the expected problems of individual electric control of the recording sites, the more serious problems with this multiwell format will be to facilitate individual fluid and pressure handling for parallel, and therefore synchronous, patching with high success rates and longevity of gigaseals, and the application of drug solutions to the recording sites when complex pharmacological tests (wash-in and washout cycles) with individual cells are required.

Due to two reasons we favor the individual, asynchronous control of each patched cell: (1) Completion of sealing and the life cycle of a stable whole-cell configuration are individual processes with a time course that cannot be predicted closely; (2) with the increase of complexity and duration of the test protocols it becomes more likely to lose cells before finishing a complete protocol. Asynchronous operation with stop-go decisions depending on control points enables the individual starting of protocols and flexible deselecting of defective sites without interference with the experiments running at other sites. This contributes to avoiding the waste of drug solution to badly sealed cells or to cells suffering from a run-down and excludes large numbers of false-negative or false-positive results due to inappropriate voltage-clamping and cell integrity. From the viewpoint of the user, high yield of gigaseals, longevity of the individual recordings, and individual control of each patched cell will result in a cost-efficient solution to typical secondary screening or assay development needs.

The CYTOPATCH™ technology is well suited for future integration of additional screening technology. There is a trend in pharmaceutical industry towards high content screening, which means questing several parameters at once on a single cell or cell culture. The first parameter is always the drug action on the ion channel activity. Typical further parameters are drug absorption, metabolization, and toxicity, mostly measured by fluorescent dyes. Fluorescent technology for single cell analysis can be added with low efforts to the CYTOPATCH™ automat because of the fixed position of the cells on the planar CYTOPATCH™ Chip.

However, progress toward realization of a prototype of the CYTOPATCH™ automat necessitates bundling of a broad range of expertise. CYTOCENTRICS has found outstanding know-how in its partners NPI Electronics (Tamm, Germany) and Multi Channel Systems MCS (Reuthlingen, Germany) for developing automated patch clamp electronic, high speed data acquisition and robotics, BionChip and Onstream MST (Eindhoven, The Netherlands) for development and large-volume production of the patch clamp chip and the NMI for cell culture, biophysical, and electrophysiological issues and nanoanalytics. Together with these partners, CYTOCENTRICS is on the way to finally fulfill the need for high quality automatic patch clamping by proofing of the capability of the CYTOPATCH™ chip to form gigaseals and whole-cell configurations with the outstanding high success rate that can be reached with the CYTOCENTERING technique.

MATERIALS AND METHODS

Polyimide Foils with Microopenings

We microfabricated openings with diameters between 8 μm and 12 μm in polymide films (thickness 6.5 μm) using standard photolithography and plasma etching. A 100 nm thick Si_3N_4 layer was deposited on top of the polyimide by use of rf-sputtering Si in Ar/N_2 plasma. On top, positive-tone photoresist Shipley S1818 is spun over the Si_3N_4 layer. This resist was structured by means of standard UV-photolithography transferring holes of desired diameter into the photo-resist layer. The latter serves as a mask when etching the Si_3N_4 layer in CF_4-plasma, resulting in a structured O_2-resistant Si_3N_4 mask for etching the polyimide in O_2 plasma. While etching the polyimide, the photo-resist is also removed. As a result, holes with the desired diameter were transferred into the polyimide with a clean Si_3N_4 layer on top.

Mechanical and Electrical Setup for Proof of Principle

The tip of a patch pipette was positioned with an accuracy of ± 1 μm in the center of an opening (diameter 8 to 12 μm) in a polyimide sheet by means of micromanipulators (LIMES 90, Owis, Stauffen, Germany) as shown in Figure 2.

The pipette was filled with pipette solution and connected via an Ag/AgCl wire soldered to a BNC connector with a single-electrode patch-clamp amplifier (EPC 7, HEKA electronic). Recordings were sampled with a software (TIDA 3.0; HEKA Electronic, Lambrecht, Germany) controlled interface board with 18 bit analog-to-digital converters (ITC 18, Instrutech). Series resistance has not been compensated.

Experiments were carried out under visual observation (custom assembled Olympus BX30MDIC with upright illumination; Olympics long distance and water-immersion objectives).

Pressure control was performed either manually with syringes or with membrane pumps (MPCU-3, LORENZ Meßgerätebau, Germany). To prevent soiling of the opening and the pipette, positive pressure was applied before filling the buffer reservoir (pipette 7 mbar, suction opening 5 mbar).

Preparation of Neuro2A Cells

Neuro2A cells are neuroblastoma-derived cells that express both Na and K channels. The cells were cultured in petri dishes in the incubator (humified air, 5% CO_2, 37°C), passaged every second day, and discarded after 10 passages. The culture medium used was DMEM with high glucose (GIBCO Cat. No. 21068-028), supplemented with 5% fetal calf serum, 2 mM glutamin, 64.8 mg/L penicillin, and 100 mg/L streptomycin. Thirty min before recording, the cells were washed with PBS and detached by standard trypsin treatment for 2 min.

Electrophysiological Recordings

During the positioning of the cells, current/voltage measurements were carried out between the two Ag/AgCl electrodes in the pipette (borosilicate glass) and in the buffer reservoir above the polyimide sheet. This allowed the observation of the seal formation during suction either by measuring the leakage current in the voltage clamp mode of the amplifier or by measuring voltage traces after current injection in the current clamp mode. From the current clamp recordings, the series resistances were calculated according to Ohm's law.

All experiments were done at room temperature using the following solutions (in mM): Bath solution 140 NaCl, 3.0 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 10 HEPES, 15 glucose; pipette solution 140 K-glutamate, 15.0 NaCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA.

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