

High Quality Ion Channel Analysis on a Chip with the NPC[®] Technology

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Abstract: In evaluating ion channel function, electrophysiology, *e.g.*, patch clamping, provides the highest information content. For the analysis of ion channel-modulating compounds, one variant of the patch-clamp technique, the whole-cell configuration, is particularly useful. We present here patch-clamp recordings in the whole-cell configuration and single channel recordings performed with planar patch-clamp chips, which are microstructured from borosilicate glass substrate. The chips are used in the Port-a-Patch[®], an ion channel research/screening instrument that enables automated patch-clamp experiments on a single cell. A software runs the experiment by executing user-determined protocols for cell positioning, as well as for electrical stimulation and current readout. In various electrophysiological experiments, the high quality of recordings and the versatility of the perfusion of the recorded cells are demonstrated. Quantitative pharmacological experiments are performed on sodium channels expressed in HEK cells using solution volumes in the low microliter range. The exceptionally low volume consumption in the experiments make the system attractive for work on rare or expensive compounds. Due to the low volumes necessary, a rapid solution exchange is facilitated, which is shown on RBL cells. The patch-clamp chip enables a rapid and precise perfusion, allowing sophisticated investigations on ion channel function with the Port-a-Patch.

Introduction

PHARMACOLOGICAL INVESTIGATIONS of ion channel modulation using electrophysiology requires means for carrying out the electrical recordings, as well as means for the application of compounds. In recent developments, the application of microstructured chips for patch clamping has been established.¹⁻³ In these experiments, the common patch pipette^{4,5} is replaced by a microperforated glass chip that enables the positioning and sealing of a cell via an automated suction protocol. This straightforward procedure allows for high-quality patch-clamp recordings, including single channels.⁶ Although the use of a planar substrate for electrophysiological experiments has a successful tradition,⁷ it is only the recent improvements that make this approach attractive for the automation and parallelization of the patch-clamp technique.

Ion channels are membrane proteins governing cellular signaling and communication by regulating the flow of charged particles (ions) into and out of cells,⁸ and several disease states can be influenced through ion channels.⁹⁻¹² Ion channels are easily accessible for drugs, because they are membrane proteins exposed to the extracellular, the intracellular, and the lipid membrane compartments alike. The metabolic activity of every cell depends crucially on the activity of its ion channels. If it is possible to tailor a drug to a particular ion channel in the membrane of one particular cell type, this cell's metabolic processes can be manipulated in a targeted manner. Thus, modification of ion channel function by ICADs is one of the most successful ways of correcting pathological deviations from normal body functions.

At present, ion channels are targeted by a number of marketed drugs directed against diseases such as epilepsy,

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ABBREVIATIONS: ICAD, ion channel active drug; IV, current-voltage; TTX, tetrodotoxin.

cardiac arrhythmia, hypertension, angina pectoris, and diabetes. However, these existing drugs target only a few distinct channel types, whereas $\sim 1.5\%$ of the human genome encodes for ion channels. Thus, the human genome project has identified >400 ion channel genes.¹³ This means that the full potential of ion channels as drug targets is far from realized today.

This backlog is caused by the fact that the only precise and reliable technique of determining ion channel activity, *i.e.*, direct, electrophysiological recording of membrane ionic currents by the patch-clamp technique, is too slow and costly to be routinely used for large volume testing in industrial drug development programs. Alternative techniques (fluorescence, flux assays) have high throughput, but are far inferior to patch clamping in terms of information content, robustness, and specificity.^{14–19} Furthermore, these techniques can only be used with a limited group of ion channels. The large-scale search for more selective, more potent, and safer ion channel modulators must use a robust screening method that delivers specific information on the mode of action.

Patch clamping has unsurpassed information content and high sensitivity and can be applied to all types of ion channels. However, conventional patch clamping requires highly trained personnel, is a laborious process, and can be used only in a sequential manner. Because of the low throughput of this technique, patch clamping is currently only applicable as a secondary or tertiary screening method, following less sensitive and more indirect approaches. Currently used ICAD screening methods are capable of high throughput, but, besides the limitations mentioned above, are known for their high

false-negative and false-positive rates. Without a cost-effective and rapid electrophysiological screening technology, large-scale R&D programs toward ICADs are uneconomical.

For these reasons, the current situation is characterized by a huge, pent-up demand for an automated, higher throughput version of the patch-clamp technology. Nanion's NPC[®] product family addresses this demand. The first instrument that uses Nanion's patch-clamp chips is the one-channel patch-clamp automat, the Port-a-Patch[®]. The Port-a-Patch is a small bench-top device, which replaces the standard patch-clamp rig. It performs automated electrophysiological experiments on a single cell at a time. Due to the rigid system design and an all electrical control, no suspension table or microscope is needed, allowing for the miniaturized design of the instrument. In Fig. 1, the Port-a-Patch is displayed.

Glass as an electrically insulating and optically neutral substrate material is the ideal choice for patch-clamp chips. The glass chips are microstructured with small perforations of a diameter of $\sim 1 \mu\text{m}$. These tiny holes mimic the geometry of an inverted patch pipette tip and allow the precise positioning of and recording from mammalian cells. The technology is scalable to a parallel patch-clamp array. A 16-channel patch-clamp screening station, the NPC-16, is in the final stages of development. The chips used with the NPC-16 contain 16 patch-clamp apertures and are easily exchanged in the screening station. The whole system is integrated in a liquid handling environment enabling automated cell and compound application and meets the demands of higher throughput applications.

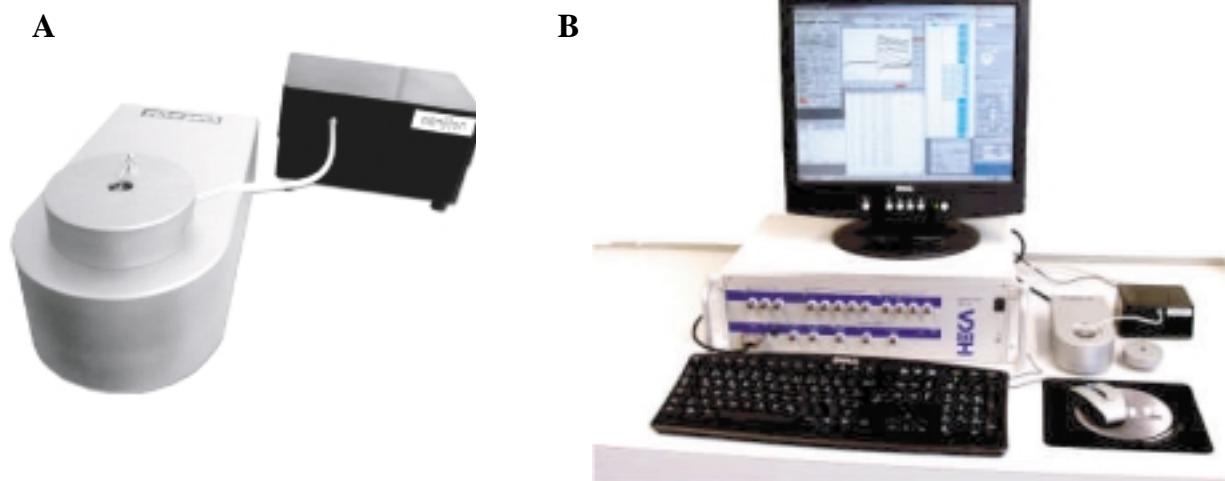


FIG. 1. The Port-a-Patch[®] ion channel screening station. (A) The chip mounting station is shown in front and the suction control unit in the back. The patch-clamp chips are supplied on small screw-on caps, which are easily mounted onto the holder enabling a quick cycling time for the chip exchange between experiments. (B) The complete Port-a-Patch setup consisting of the chip holder and suction control, an EPC-10 amplifier, and a computer. This setup basically replaces the common patch-clamp rig.

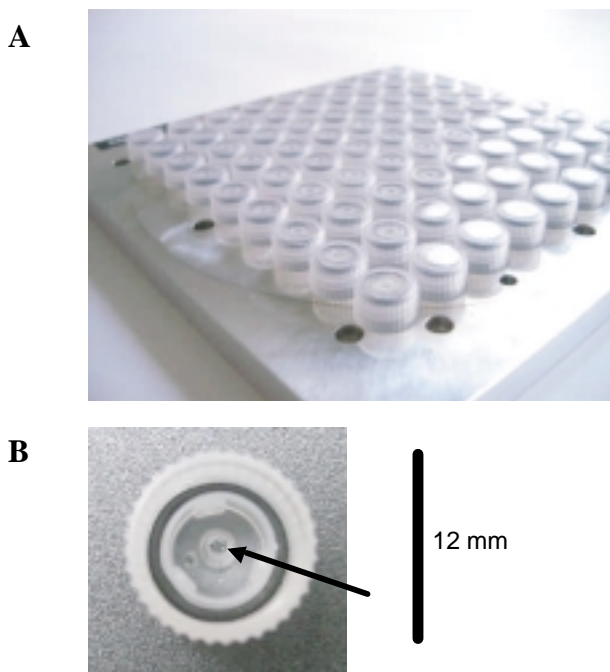


FIG. 2. The patch-clamp chips made from borosilicate glass substrate (A). The chips are glued onto small caps, which are easily mounted in the Port-a-Patch[®]. The caps have a small cone on the backside of the chip (B), which is filled with intracellular solution (see arrow). An electrode in the holder reaches into this solution-filled cone, making an electrical contact with the chip.

Materials and Methods

Cell preparation

Experiments were performed using CHO cells, HEK cells, and RBL cells. The cells were either used as wild-type cells or stably transfected with various types of ion channels. The culture conditions used were the standard conditions described in the ATCC catalogue for each cell line.

Optimal results were obtained by splitting the cells every other day. The confluency of the cells before harvesting for the experiment should be in the range of 50–80%. Cells are prepared in suspension for the electrophysiological experiments. For detaching the cells before the experiment, no significant difference in performance could be found by using either trypsin Accutase, enzyme-free solution or a cell scraper, but some ion channels lose the functionality by using enzymes for cell detachment. After the detachment, the cells were washed two times with the external measuring solution. The optimal cell density of the suspension ranges from 5×10^5 to 5×10^7 cells/ml. The cells were kept at room temperature and mixed with a 1-ml pipette before the measurement.

Chip fabrication and handling

Borosilicate glass with a thickness of 100 μm was used as a substrate for the chips. Well-type structures were thinned into the glass, leaving a glass membrane with $\sim 20 \mu\text{m}$ remaining thickness. Into this membrane, a conical-shaped perforation is introduced. The details of the preparation are described elsewhere.^{2,6}

The disposable consists of a screw-cap onto which the glass chip is glued. This design allows a fast exchange of the chip after each experiment and enables pharmacological experiments with low volume consumption. Figure 2 shows the NPC-1 glass chips, which are filled with electrolyte solution and mounted onto the holder.

The disposables are packaged for protection against moisture and contamination. After removing the packaging from the disposable, we have found it advantageous to use the chips within 1 week. Typical chip parameters are listed in Table 1.

Experimental procedure

The chip is first filled from the bottom side (the side with the screw thread). For bubble-free filling, it is advisable to use a thin tip, which is typically used for pipette filling (e.g., MicroFil, MF34G-5, WPI). Eight microliters of a sterile filtered solution is sufficient to fill the cone. After the chip is screwed on top of the holder, the top (glued glass chip) can be “filled.” Five microliters of a sterile filtered solution is adequate to wet the glass chip. Both solutions need to contain Cl^- , as it is the conducting ion. The chloridized electrode is prepositioned to reach into the liquid and serve as the ground electrode.

Five microliters of a cell suspension is placed on top of the chip. After the addition of the cell suspension, a gentle suction has to be applied to place the cell onto the aperture. In contrast to the classical patch-clamp technique, it is the cell that is moved to the aperture. The development of a seal on top of the chip is slightly different from the seal of a cell with the tip of the pipette. The seal develops more continuously with a resistance up to several gigaohms. Then the cell membrane on top

TABLE 1. TYPICAL CHIP PARAMETERS

• Chip resistance:	$5.7 \pm 2.2 \text{ M}\Omega$, $n = 300$
• Chip capacitance:	$<1 \text{ pF}$
• Seal resistance:	$>1 \text{ G}\Omega$
• Access resistance:	6–15 $\text{M}\Omega$
• Whole-cell resistance:	$>200 \text{ M}\Omega$
• Whole-cell recording:	HEK 293 cells: 30–50% CHO cells: 30–50% RBL cells: 50–70%
• Whole-cell stability	$>30 \text{ min}$
• Full dose-response curve possible on one cell	

of the aperture can be ruptured by a sudden increase of suction, like in a conventional patch-clamp experiment. The result is also an electrical connection to the inside of the cell.

If a seal resistance of a gigaohm is not achieved, a continuation of the measurement is still reasonable. In a conventional patch-clamp experiment, there is a significant drop of the seal resistance by getting access to the whole cell, not seldom down to 200 M Ω . This is not the case when the NPC-1 chip is used. The resistance usually remains constant during the process of going to the whole cell; after reaching the whole cell and just maintaining the access, the seal resistance increases again.

In case of an increase in series resistance, it is advisable to apply again some suction. It is sometimes helpful to keep a gentle suction during the experiment to keep the cell from resealing. This is also known for conventional patch-clamp experiments and can be adjusted in the PatchMaker software supplied by Nanion.

For some cell lines, it tends to be difficult to rupture the membrane for obtaining whole-cell access. In this case, it

appears to be helpful to use a zapping pulse in addition to some suction. This method works with the NPC-1 chip more reliably than with a conventional patch pipette. In our hands, it worked best to use the highest voltage the amplifier can deliver (1 V) and a short pulse duration (500 μ s). In case no reaction of the cell is seen during the zapping pulse, the pulse duration can be increased.

Electrophysiology

The Port-a-Patch system used for the experiments consists of the suction control unit and a chip mounting station and uses an EPC-10 amplifier (EPC-10, HEKA, Germany). The recorded data were filtered at 3 kHz and sampled at 10 kHz. The electrolyte solutions used had the following ionic compositions (mM): extracellular (top of chip): NaCl (140), KCl (3), MgCl₂ (1), CaCl₂ (1), HEPES (10), pH 7.4; intracellular (underside of chip): for HEK cells: CsCl (130), NaCl (10), HEPES (10), EGTA (10), pH 7.2; for CHO cells: KCl (120), NaCl (10), EGTA (10), HEPES (10), pH 7.2; for RBL cells, extra-

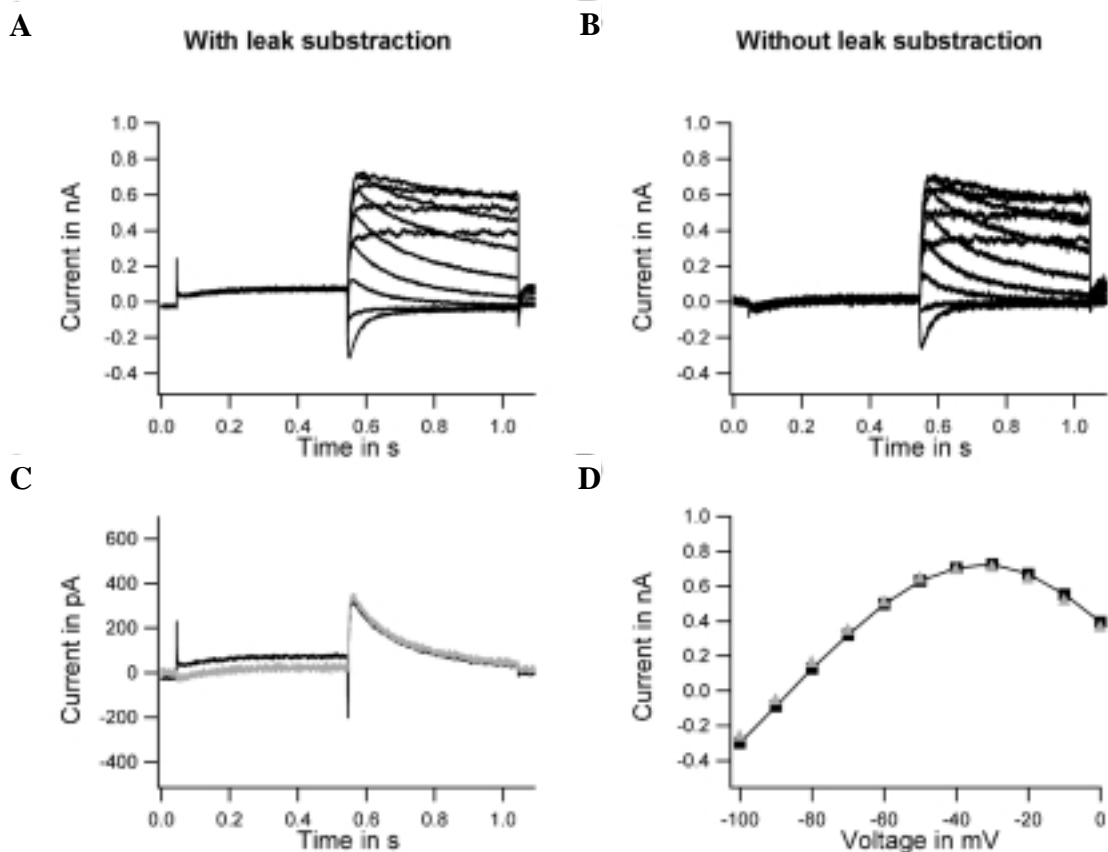


FIG. 3. Whole-cell recordings performed with the Port-a-Patch[®]. CHO cells expressing the hERG channel are recorded with a standard voltage protocol for hERG. (A) Typical data obtained with the Port-a-Patch are displayed without leak subtraction (e.g., P/n pulses) applied. (B) Recordings from the same cell with leak subtraction activated are displayed. (C) Comparison of the recorded traces with and without leak subtraction for the voltage step from +40 mV to -40 mV. (D) The IV curves for the data from A and B are plotted for comparison. The comparison of the obtained data shows the high quality of the recordings.

cellular: NaCl (123), KCl (20), MgCl₂ (1), CaCl₂ (1), HEPES (10), pH 7.4; intracellular: potassium aspartate (120), EGTA (10), HEPES (10), NaCl (10), pH 7.2.

Results

In Fig. 3, whole-cell recordings from CHO cells performed with the Port-a-Patch are shown. The CHO cells express hERG channels, and a standard hERG channel voltage stimulation protocol is applied. The current response to the voltage protocols and the corresponding IV relations are shown. Recordings from the same cell with (Fig. 3B) and without (Fig. 3A) leak subtraction are displayed, to demonstrate the high quality of the patch-clamp recording. The current data and the IV curves have the typical characteristics of the hERG channel.

Although the NPC-1 chip has a simple design, it is very suitable for pharmacological measurements. Due to the minute volume of solution on the chip, only tiny amounts of compounds need to be applied for their pharmacological evaluation. To demonstrate the excep-

tionally low compound consumption and the ease of performing precise pharmacological investigations, dose-response relations of TTX applied to rNav1.2a channels expressed in HEK cells have been measured. A known volume with known number of cells in suspension is pipetted onto the chip and, after the whole cell configuration has been established, the appropriate amount of a stock solution containing the TTX is added to the chip. For increasing concentrations, either a more concentrated stock solution can be added, or part of the volume on the chip is removed with a pipette and replaced by more TTX-containing solution. Both procedures are reliable and precise. In Fig. 4, whole-cell recordings from the HEK cells expressing the sodium channel and the IV curve for the recording are shown. The data from the pharmacological experiment are also displayed in a dose-response curve, resulting in an IC₅₀ of ~15 nM for TTX acting on the sodium channels (literature value 12 nM²⁰). Even these rapidly gated channels can be easily investigated with the Port-a-Patch.

Solution exchanges and test compound applications can be performed very rapidly. In Fig. 5, whole-cell

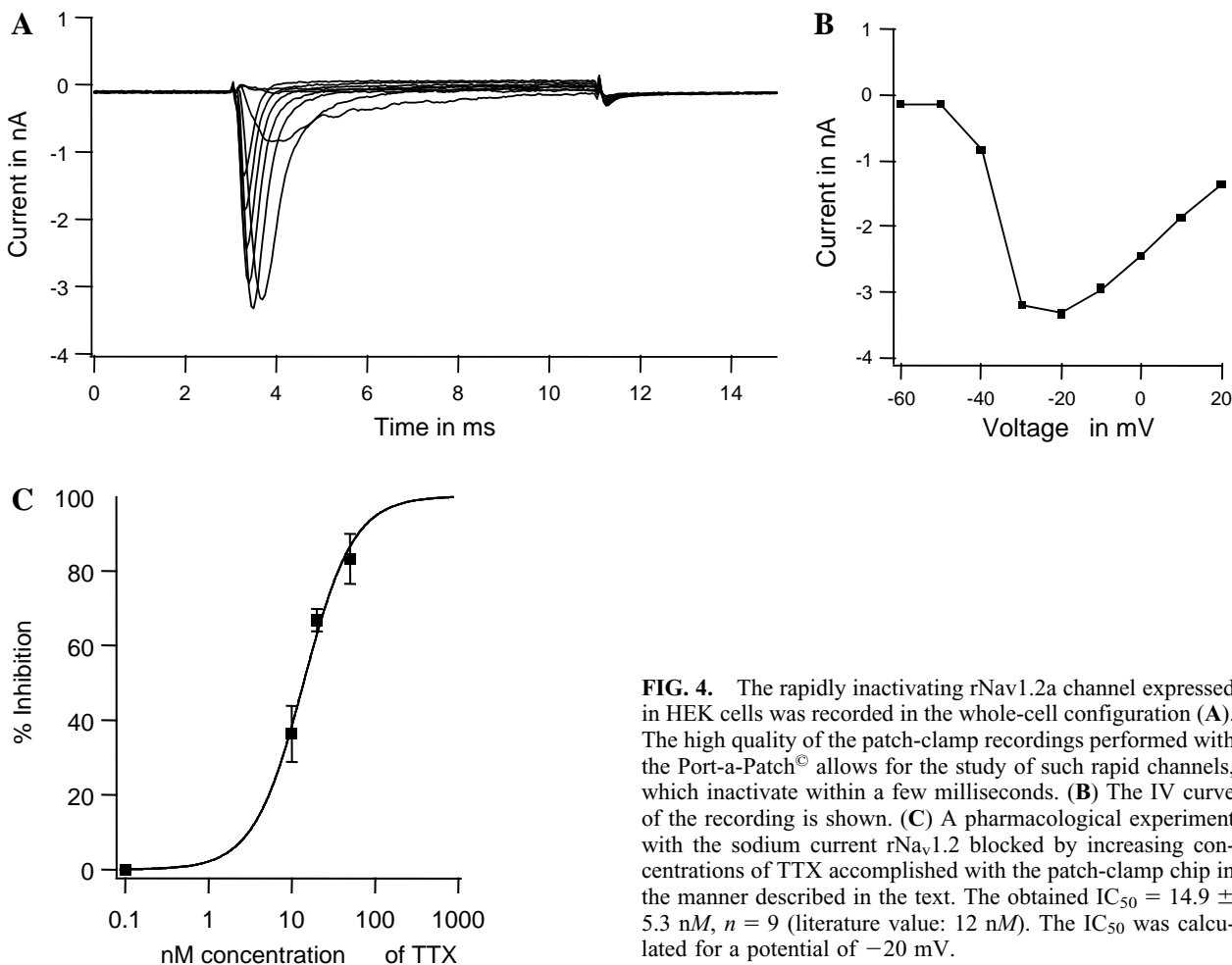


FIG. 4. The rapidly inactivating rNav1.2a channel expressed in HEK cells was recorded in the whole-cell configuration (A). The high quality of the patch-clamp recordings performed with the Port-a-Patch[®] allows for the study of such rapid channels, which inactivate within a few milliseconds. (B) The IV curve of the recording is shown. (C) A pharmacological experiment with the sodium current rNa_v1.2 blocked by increasing concentrations of TTX accomplished with the patch-clamp chip in the manner described in the text. The obtained IC₅₀ = 14.9 ± 5.3 nM, n = 9 (literature value: 12 nM). The IC₅₀ was calculated for a potential of -20 mV.

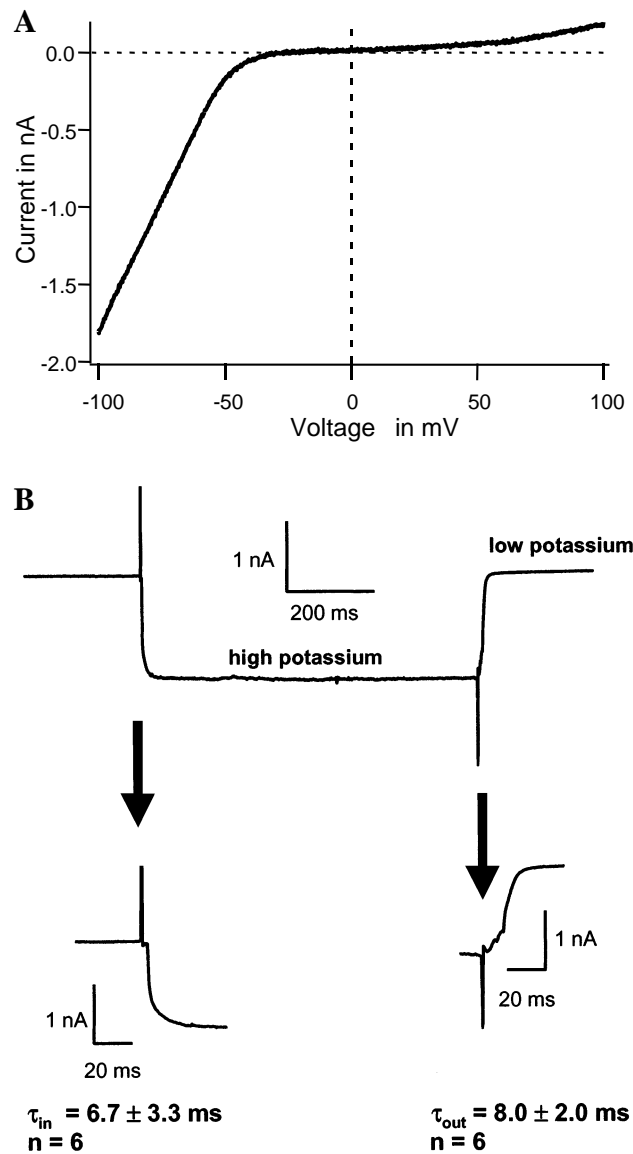


FIG. 5. Rapid perfusion of RBL cells patch-clamped with the glass chip. (A) The IV relation of the RBL cells is displayed, which exhibits the characteristics of the endogenously expressed inward rectifying potassium channels. The potassium current of the RBL cells voltage-clamped at -100 mV is then used to demonstrate the rapid perfusion. By varying the potassium concentration on the extracellular side, the whole-cell current is increased and decreased correspondingly (B). By decreasing the potassium concentration, the current drops by >1 nA and fully recovers upon increase of the potassium concentration to the former level. The τ for the perfusion is <10 ms, making the Port-a-Patch suitable even for very rapid pharmacological experiments.

recordings from rapidly perfused RBL cells are presented. The RBL cells endogenously express inward rectifying potassium channels and the IV relation measured with the Port-a-Patch is shown in Fig. 5A. For the perfusion experiment, the cells were voltage-clamped at -100 mV, and the potassium concentration in the exter-

nal solution was rapidly varied. The addition of a high concentration of potassium solution increased the conductivity as more ions could permeate the channels. The resulting increase in the holding current is completed within <10 ms. Reducing again the external potassium concentration results in the reverse effect. A complete perfusion cycle is shown in Fig. 5B. The resulting τ for increasing and decreasing the potassium concentration is $\tau_{in} = 6.7 \pm 3.3$ ms and $\tau_{out} = 8.0 \pm 2.0$ ms. The fast perfusion makes the planar patch-clamp approach applicable for studying even fast inactivating ligand-gated ion channels.

With the Port-a-Patch, it is also possible to carry out experiments on single ion channels. This is not in high demand for screening applications, but simply shows the quality and performance of the technology. Single channel recordings can be performed in the cell-attached configuration, where the cell is positioned and sealed on the aperture in the chip, but the cell membrane is still intact and not ruptured for whole-cell access. In this configuration, the recordings from the ion channels only in the

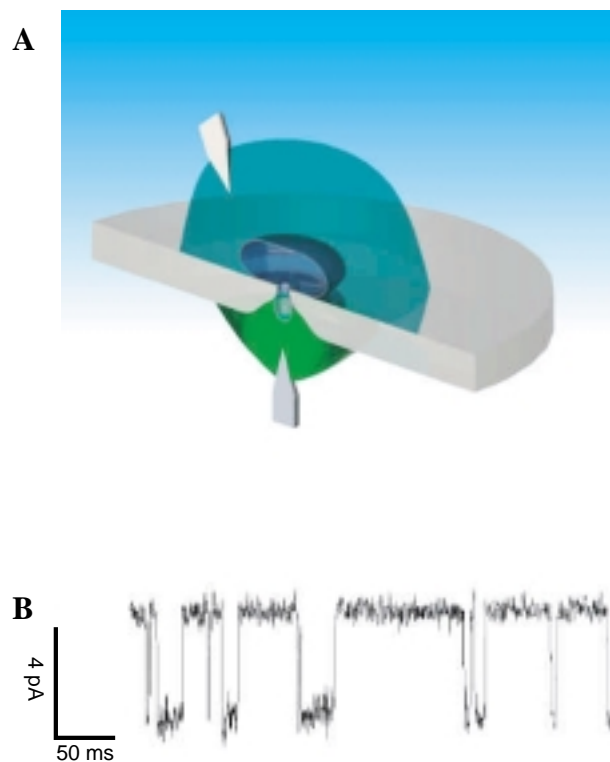


FIG. 6. Single channel recordings from BK channels expressed in a CHO cell. A cell is positioned and sealed onto the aperture in the chip, and the voltage clamp of the membrane patch spanning the aperture allows the recording from only the ion channels in this part of the membrane (cell-attached configuration). (A) Schematic of the cell-attached configuration on the chip. (B) Single BK channel current trace of a CHO cell voltage-clamped at -70 mV in the cell-attached mode. The data are filtered at 1 kHz.

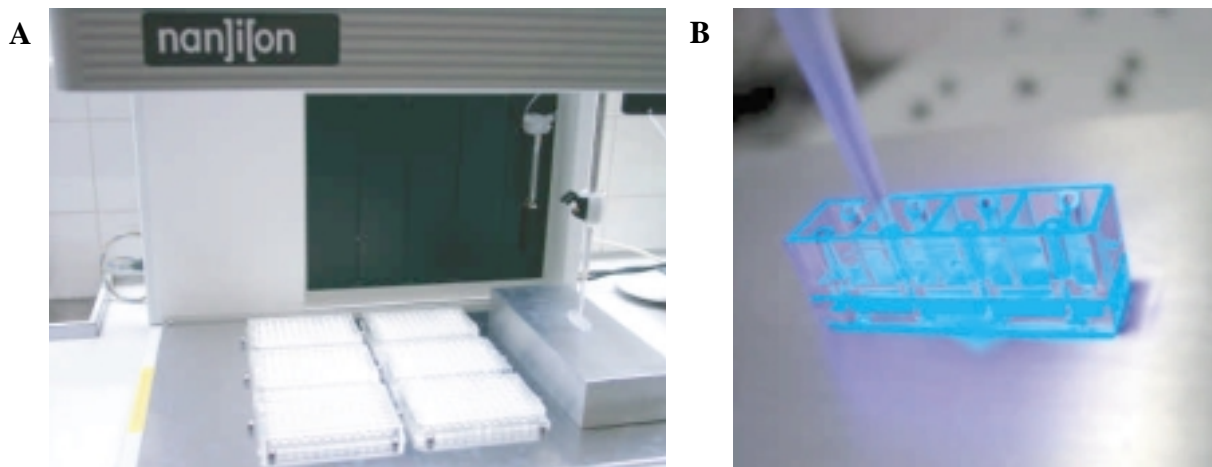


FIG. 7. The prototype four-channel NPC[®] workstation for automated electrophysiology with the disposable patch-clamp NPC chips.

very membrane patch across the aperture are possible. In Fig. 6, single channel recordings from a CHO cell expressing the BK potassium channels are shown. These recordings were performed in the cell-attached mode, which basically is the default situation after positioning and sealing of a cell.

The NPC-16: toward higher throughput

The positioning of cells for patch-clamp recording with simple suction applied to the chip lends itself to automation and parallelization. The opportunity of parallelizing electrophysiological characterization of drug can-

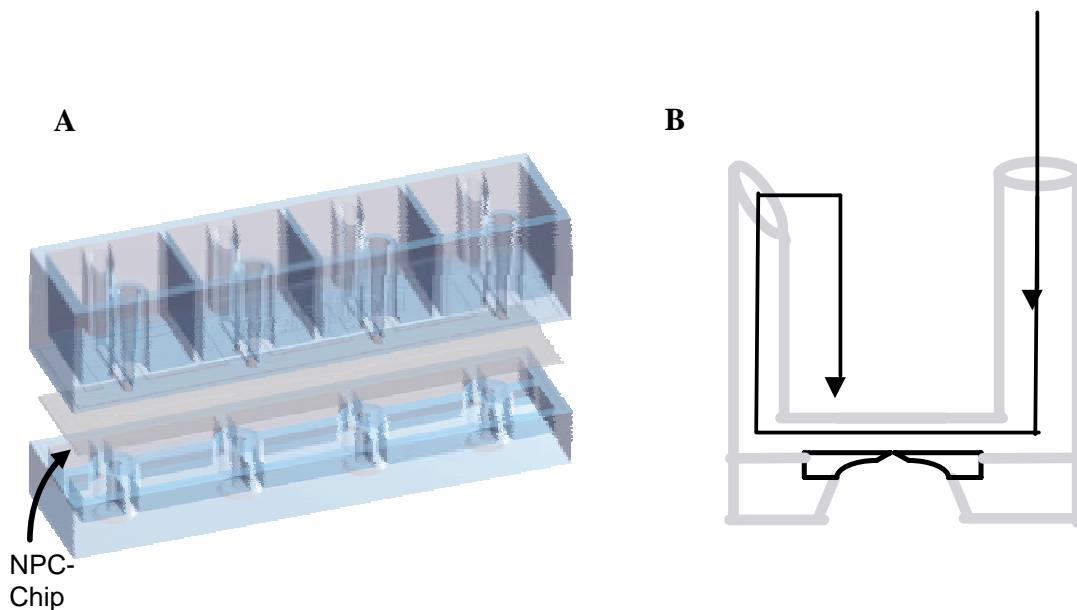


FIG. 8. The NPC[®] microfluidic perfusion cartridge (A). The cartridge enables in a pipetting robotic compatible manner very rapid and precise perfusion of the recorded cell with very low solution/compound volume consumption (<15 μ l). The cartridge contains inlets, in which standard pipette tips (200 μ l) are inserted by the robot. The robot withdraws the liquids to be applied from microtiter plates and perfuses the microfluidic channels containing the patch-clamp unit with solution via the inlets. The liquid is pressed through the channel by the robot, and the solution spills into a waste reservoir (central square compartment) via the outlet. The depicted cartridge contains four patch-clamp perfusion units. The path of the electrolyte/compound solution is indicated in the schematic on the right (B).

didates and drug target channels makes our approach attractive for high throughput drug screening.

To address the needs for increased throughput of ion channel screening, we have developed a fully automated electrophysiology screening station, called NPC-16. It uses a chip disposable that comprises 16 patch-clamp units in an array format. For each of the 16 patch-clamp apertures, an individual suction control line is supplied. The complete suction control unit and all electronics are integrated into a pipetting robot. The chips are inserted, and the workstation applies solutions, cells, and compounds according to a predefined protocol and carries out the electrophysiological measurement in each patch-clamp unit. Hence, the liquid handling, the cell application, and the experimental protocol are fully automated. We estimate that the NPC-16 allows the measuring of up to 400 compounds per day. For five different compound concentrations, this adds up to 2,000 data points per day. The four-channel prototype of this screening station and the perfusion cartridge are displayed in Fig. 7.

The NPC-16 chips are equipped with a system for rapid perfusion of the cells with test solutions. This is realized by a microfluidic cartridge, into which the NPC-16 chips are embedded. The cartridge contains docking stations for disposable pipette tips, so that standard pipetting robots can be used in the operation of the device. This combination of microfluidics with standard pipetting robotics enables automated and repetitive application of compounds, which are applied from microtiterplates. The integrated perfusion system allows for rapid and accurate solution exchange, as well as for in-flow measurements. In Fig. 8, the microfluidic cartridge and the perfusion principle are shown in a four-well design used as a prototype.

The high speed of solution exchange (<50 ms) is of great value for the study of ligand-gated ion channels. The NPC-16 chips use small volumes of test solutions of <15 μ l per concentration ("lab-on-chip" approach), hence minimizing the consumption of test sample volume. This is of extraordinary importance, especially for rare and expensive substances.

Discussion

The common handmade patch pipette is replaced by a microstructured glass chip for cell positioning and analysis. Instead of positioning a pipette onto a cell with a micromanipulator under visual control (microscope), cells are positioned on the apertures in the chip by simple suction. The suction used to position the cells is controlled by electric valves and automatically regulated by a feedback loop according to an electrical readout of aperture resistance. Cells in suspension are simply pipetted

onto the patch-clamp chip and using a software-controlled suction protocol, the whole-cell configuration of a single cell is automatically established for the electrophysiological experiment. This drastically reduces the equipment and scientific skills needed for a common patch-clamp setup, as no microscope, micromanipulator, and vibration isolation table are necessary. The scaled up array format enables automated, rapid, and parallel screening of ion channel-modulating drugs.

A first step toward electrophysiological screening was the automation of two-electrode voltage-clamp recording of *Xenopus* oocytes, which is now extended to voltage clamping of mammalian cell lines by various companies.^{3,17} The automation of the patch-clamp technique and the resulting increase in throughput capability will have a significant impact on the ion channel drug discovery process and ion channel research in general. It will decrease the time needed for ion channel drug discovery, reducing costs and time-to-market for ICADs. It also enables large-scale safety pharmacology screening (e.g., for hERG modulation) using electrophysiological methods with all their advantages.

The presented technology allows for sophisticated experimental protocols, including the study of use and voltage dependence of compounds providing high time resolution for fast activation/inactivation kinetics. This enabling technology will be a valuable tool for anyone involved in the research and development of ion channel drug targets.

Acknowledgments

The CHO cells expressing the hERG channel were kindly provided by Iongate GmbH (Frankfurt, Germany), the HEK cells expressing the sodium channel by Neurosearch A/S, Copenhagen, Denmark), and the CHO cells expressing the BK channel by the 4SC AG (Martinsried, Germany).

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