Development of a Novel Automated Ion Channel Recording Method Using “Inside-Out” Whole-Cell Membranes

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Efforts to develop novel methods for recording from ion channels have been receiving increased attention in recent years. In this study, the authors report a unique “inside-out” whole-cell configuration of patch-clamp recording that has been developed. This method entails adding cells into a standard patch pipette and, with positive pressure, obtaining a gigaseal recording from a cell at the inside tip of the electrode. In this configuration, the cell may be moved through the air, first rupturing part of the cellular membrane and enabling bath access to the intracellular side of the membrane, and then into a series of wells containing differing solutions, enabling robotic control of all the steps in an experiment. The robotic system developed here fully automates the electrophysiological experiments, including gigaseal formation, obtaining whole-cell configuration, data acquisition, and drug application. Proof-of-principle experiments consisting of application of intracellularly acting potassium channel blockers to K⁺ channel cell lines resulted in a very rapid block, as well as block reversal, of the current. This technique allows compound application directly to the intracellular side of ion channels and enables the dissociation of compound activities due to cellular barrier limitations. This technique should allow for parallel implementation of recording pipettes and the future development of larger array-based screening methods. (Journal of Biomolecular Screening 2005:806-813)

Key words: potassium channels, screening, patch clamp, intracellular

INTRODUCTION

ION CHANNELS ARE MEMBRANE PROTEINS containing multiple potential extracellular binding sites through which pharmacological agents can act. In practice, however, many compounds have been identified that block the ion flow via binding to the intracellular vestibule of the channel. Compounds must thus diffuse through the cell membrane to reach their target, resulting in the elimination, due to cellular impermeability, of many potentially active compounds at the earliest stage of screening. Thus, if compounds could be applied to the intracellular face of the membrane, many more structural compound classes could be identified with relevant pharmacological activity in the early stages of screening. In addition, more accurate compound kinetics could be obtained with intracellular application, as membrane diffusion is not a confounding factor in the measurement of the structure-activity relationship. Subsequent refinement of compound structures would then be used to address slow or incomplete diffusion through the cellular membrane, with the ultimate goal of identifying compounds that are active when applied in the extracellular solution to whole cells.

Discovery of novel drugs that modulate the electrical excitability of the cell has been hindered in the past in part by the lack of a technology that enables high-throughput screening (HTS) of potential ion channel modulators. Numerous indirect methods are routinely used in ion channel drug discovery (voltage- and calcium-sensitive dyes, radioisotopes, and Rb⁺ efflux), resulting in numerous false-positive hits and false negatives and a general failure to provide accurate pharmacological constants for ion channel modulators. Moreover, many potentially active compounds are eliminated at the earliest stage of screening simply because of a slow diffusion rate through the cellular membrane. Although patch-clamp recordings remain the gold standard in the field of ion channel drug discovery, their use in drug screening is limited by several factors. Micromanipulation of living cells, complicated drug delivery, data acquisition, and analysis are time-consuming processes involving substantial human intervention. In addition, the rate of output does not approach high- or medium-scale screening standards, thus severely limiting the number of novel compounds that can be examined.

A number of recording systems have recently been described for screening of ion channels. The Flyion® system uses a recording configuration in which cells are placed inside a glass pipette, negative pressure is applied from outside the tip of the electrode, and pharmacological agents are perfused inside the pipette, an of-
ten difficult operation. Another system based on glass pipettes is the “Interface-Patch” system used by Xention, Inc. (formerly CeNeS). This automates many of the same processes employed by conventional patch-clamp technologies, but without the need for imaging cells under study. Multiple reports have described the development of silicon-, polyethylene-, PDMS-, quartz-, Teflon-, or polymer-based planar arrays (patch-on-a-chip) with holes mimicking pipette tips, although each technique has its strengths and weaknesses, as reviewed previously.11-13 Polymer arrays are available commercially (Molecular Devices), but GOhm seals onto cell membranes have not been possible to date, thus limiting its acceptance by the ion channel community. Quartz and silicon substrates, however, are perhaps more promising, as GOhm seals comparable to conventional pipettes are possible.7 This technology is still developing, however, and thus it is difficult to predict which technique will ultimately be successful.

Here we describe a novel robotic system for ion channel drug screening, using a new “inside-out” whole-cell patch-clamp configuration. This configuration makes possible fast application of agents directly onto the intracellular membrane, without the need for a complex pipette perfusion apparatus. Compound diffusion through the cellular membrane is not required, yet the advantage of large current sizes is retained, unlike with excised inside-out membrane patches. The robotic system described enables a minimum of human intervention and potentially provides a new medium-throughput screening method for ion channels.

METHODS

Robotic patch-clamp station (RoboPatch)

A picture of the RoboPatch main operational unit is presented in Figure 1A, and an operational diagram of the RoboPatch automated patch-clamp station is drawn in Figure 1B. An interface program (VB6.0) receives operational parameters and synchronizes the operation of all RoboPatch units—EPC9 amplifier, 3D robot, peristaltic pump, and a pressure regulator. Pipette resistance is used as a parameter to monitor cell membrane–glass tight seal formation, as in the traditional patch clamp. The pipette resistance was monitored using the EPC9 amplifier and exchanged by a file-sharing communication protocol between Pulse and the interface program. A pressure regulator adjusted the positive pressure inside the patch pipette in accordance with current resistance values. Pressure was altered in a pattern similar to that used with the manual patch-clamp method, with the exception that positive pressure was substituted for negative pressure. The algorithm was repeated in cycles at 5 to 10 Hz until gigaseal formation occurred.

The 3D positioning axes were purchased from Velmex, Inc. (8-μm step precision, Bloomfield, NY). Velmex 3D stage movement was controlled by the RS232 communication protocol integrated into the VB6.0 environment.

Pressure inside the patch pipette was controlled by a compact pressure regulator (QB1, Proportion-Air, McCordsville, IN). The

![FIG. 1. Principle of operation of the robotic patch-clamp (RoboPatch). (A) Picture of RoboPatch with 96-well plate compound application system. Labels indicate the main system components, plus some optional components (single wash well, cell reservoir, and cell mixer) that were used for some experiments: (1) pipette holder, (2) pipette gripper, (3) cell reservoir, (4) recording pipette and reference electrode, (5) washer, (6) pressure line, (7) compound plate, (8) Faraday cage, and (9-11) x, y, and z axes. (B) Schematic representation of connections between RoboPatch operational units, including an interface program, 3D moving platform, HEKA patch-clamp amplifier, and a pressure controller. The value of current through a patch pipette is monitored by the HEKA Pulse data acquisition system and sent to the interface program at 0.5-sec intervals via a file-sharing protocol. The pressure controller receives a command to switch pressure to zero differential when the pipette series resistance reaches a predefined (user-determined) value. The interface program synchronizes the 3D robot movement and HEKA data acquisition protocol.

Electrophysiology

Currents were recorded using a novel inside-out whole-cell technique. Chinese hamster ovary (CHO)–K1 cells expressing Kv4.3/KChIP1 or hERG (constructed using standard methods) were injected manually into a standard patch pipette, although this process is amenable to automation. A steady pressure in the range of 0.7 to 3 psi differential was maintained inside a patch pipette by a computer-controlled pressure regulator. A pressure differential
forced cells to drift toward the pipette tip opening (Fig. 2A-C) that eventually (5-15 sec) led to gigaseal formation. Pipette resistance was monitored at 5 to 10 Hz using a file-sharing protocol between Pulse and an interface program (Visual Basic 6.0, written locally). The pressure differential was switched to zero after gigaseal formation. A whole-cell inside-out configuration was obtained by briefly exposing the pipette tip to the atmosphere. The RoboPatch interface program detected membrane/seal resistance, whole-cell transition and controlled the air pressure applied to the pipette. Electrodes were pulled from borosilicate glass capillaries (TW150-4; World Precision Instruments, Sarasota, FL) using a conventional pipette puller. Pipette resistances were 2 to 3 MΩ when filled with Hanks balanced salt solution (HBSS). The intracellular (bath) solution consisted of (in mM) 130 KCl, 20 HEPES, pH adjusted to 7.4 with Tris-HCl. The extracellular (pipette) solution was HBSS (Cat. 14715-103, Gibco). HBSS contained (in mM) 138 NaCl, 2 CaCl2, 1 MgCl2, 0.34 Na2HPO4, 5.3 KCl, 0.44 KH2PO4, and 5.6 dextrose. Data were collected using a HEKA EPC9 amplifier and digitized using an ITC16 interface and Pulse software (HEKA Electronik, Lambrecht, Germany). Standard capacitance and series resistance compensation were used via the Pulse software. Current traces were filtered at 1 to 2 kHz and digitized at 5 to 10 kHz. Recordings were made at room temperature (22-23 °C). Data were analyzed using Pulsefit and Origin 7 (OriginLab, Northampton, MA).

Cell preparation

Kv4.3/KChIP1 or hERG stably expressing CHO-K1 cells were dissociated from a 75-cm² flask by a standard trypsinization procedure, further diluted into 50 mL of growth media, and subsequently centrifuged at 200g for 5 min. The pellet was resuspended into HBSS and gently triturated to reach a final single-cell suspension at a concentration of 0.1 to 0.3 million cells/mL.

RESULTS

Inside-out whole-cell patch clamp

CHO-K1 cells stably expressing Kv4.3/KChIP1 were injected into the patch pipette at a concentration of 0.1 to 0.3 million/mL, suspended evenly in pipette solution. A positive pressure of 0.7 to 3 psi differential was maintained inside the patch pipette by a pressure controller, which forced cells to drift toward the pipette tip. At the moment a cell reached the pipette tip (Fig. 2A), the pipette resistance began to increase, as detected by the interface program. This combination of cell density, pressure, and a single-cell suspension resulted in 1 cell reaching the pipette tip within 10 to 20 sec. Further movement of cells toward the pipette tip was not observed once this point was obtained. The pressure controller set the pressure to zero differential when a tight electrical junction (gigaseal) between the cellular membrane and the glass surface
formed (Fig. 2B). Whole-cell inside-out configuration was established by exposing the pipette tip to the atmosphere or by applying several short pressure pulses (Fig. 2C). The success rate of the above-mentioned protocol for true gigaseal and subsequent whole-cell formation was in the range of 70% to 80% for these cells.

Figure 3A shows an equivalent electrical circuit of the recording system. The ion current through the cellular membrane is driven by membrane voltage \( V_M \) that represents a difference between the applied voltage \( V_A \) and voltage drops through serial resistances \( R_1P \) and \( R_2P \). Under DC conditions, \( V_M = V_A - I_M (R_1P + R_2P) \). The tight junction resistance between the cellular membrane and patch pipette (leakage resistance) was on the order of GOhms and could be excluded from consideration. Thus, the inside-out whole-cell configuration can be considered similar to the conventional whole-cell configuration with the above-mentioned notion of distributed series resistance.

**Ion current recordings**

During the process of exposing the pipette tip to the atmosphere, it became apparent that the cellular membrane inside the pipette was not adversely affected by repeated exposures to air. Thus, it became possible to move the pipette tip with a cell through a series of wells (standard 96- or 48-well plates were used) containing different compound solutions, using the RoboPatch software to control the movements (Fig. 3B). Cells were usually stable for >30 transfers, thus allowing screening through a number of different compounds/concentrations. The reference electrode moved together with the recording electrode, and both were washed in control solution between different compounds.

One of the main concerns in ion channel drug screening is stability of the electrophysiological recordings. We tested the performance of the RoboPatch system in a well-characterized CHO-K1 cell line that possesses minimal endogenous conductances. Ion currents were recorded from CHO-K1 cells expressing Kv4.3 along with the accessory subunit KChIP1. Outward potassium currents were elicited from a holding potential of –70 mV to a test voltage of 40 mV, preceded by a 500-msec-long prepulse to –110 mV. Representative current traces from a single cell obtained at 5-min time intervals are shown in Figure 4A. Recordings showed stable peak current amplitude (Fig. 4B) and stable series resistance (data not shown) over tens of minutes. A noticeable change in current kinetics (unrelated to this recording method) reflects an endogenous property of the Kv4.3 channel and has been observed in traditional inside-out patch-clamp recordings.

**Compound application**

Compound application directly to the inside of the cell was performed by moving the pipette tip into a well containing a desired compound. Using the Kv4.3/KChIP1 cells and the well-known blocker 4-AP, we observed a fast onset and washout of compound action, consistent with a high diffusion rate in the inside-out whole-cell configuration. A family of current records of a selected cell depolarized to various voltages is shown in Figure 5A, left panel. Bath (intracellular solution) application of 10 mM 4-AP blocked the current amplitude by 53% at the first set of depolarization pulses. The effect was fully reversible by moving the pipette tip/cell back into control solution (Fig. 5A, right panel). In another cell, a pharmacological block of Kv4.3/KChIP1 channels by 4-AP reached a steady-state value within the 10-sec interval between pulses and was almost fully reversible within a similar time period.
The detailed time constant of 4-AP action was not studied at higher time resolutions.

We also used a CHO-K1 cell line expressing hERG channels to characterize a known slow-acting intracellular blocker, E-4031. In the traces shown in Figure 6, pharmacological block of hERG current by 10 µM E-4031 reached a steady-state value within the 30-sec interval when the cell was moved from control to compound-containing solution (Fig. 6B).

The hERG cell line was also used to screen a random set of compounds (Fig. 7). Moving cells between wells containing control solutions established the level of current “rundown” (about 20% in 5 min) inherent in this cell line. Comparing these control data to compound-containing wells demonstrates a clear separation when a strong blocker is encountered (Fig. 7B). This blocker was not easily reversible; thus, data from compounds in wells 8 to 14 were not obtained in this experiment.
**FIG. 7.** Screening random compounds on hERG channels can identify active molecules. (A) hERG current traces in control solution (compound 0) or the active compound 7. Holding potential was –50 mV, and a 2-sec prepulse to –100 mV was applied before the test potential. Currents were elicited by a 1-sec-long test pulse to 40 mV, followed by a “tail” voltage step to –100 mV. One test pulse was applied every 10 sec. The 3 traces for control and compound 7 are shown. (B) Using the protocol in (A), 14 compounds or controls were tested on each cell. Tail current amplitudes are plotted versus time for control wells (solid squares, n = 8) and compound-containing wells (solid circles, n = 2). Three data points were collected in each compound well. Data represent the mean ± SE. All compounds were tested at 10 µM. The Z' factor comparing compound and control wells was calculated and plotted.

**FIG. 8.** hERG channel biophysical parameters did not differ when measured by the conventional whole-cell and inside-out whole-cell methods. Families of hERG potassium currents recorded in whole-cell (A, upper traces) and inside-out whole-cell (A, lower traces) modes. Holding potential was –80 mV. Currents were elicited by 1-sec-long test pulses ranging from –60 to 60 mV in steps of 20 mV, followed by a 0.5-sec voltage step to –100 mV. (B) G/ Gmax was calculated from peak tail currents (inset) normalized to the tail current obtained from the test pulse to 60 mV. G/Gmax relationships for whole-cell (solid squares, n = 7) and inside-out whole-cell (solid circles, n = 4) were fitted with Boltzmann equations. The parameters for hERG steady-state activation were V1/2 = 17.8 ± 0.8 mV, k = 17.1 ± 0.6 mV, and V1/2 = 12.9 ± 1.7 mV, k = 16 ± 1.4 mV for whole cell and inside-out whole cell, respectively.
Comparison of inside-out and traditional whole-cell patch clamp

To further characterize the inside-out whole-cell method, we compared kinetic and steady-state parameters for recombinant hERG channels expressed in CHO-K1 cells using inside-out and traditional whole-cell recordings. We did not find any significant difference in hERG channel activation or deactivation kinetics (Fig. 8A), hERG current reversal potential (data not shown), or steady-state activation (Fig. 8B) when comparing the conventional and inside-out whole-cell patch clamp. The hERG channel steady-state activation G/Gmax, calculated from the normalized peak amplitudes of tail currents, was V1/2 = 17.8 ± 0.8 mV, k = 17.1 ± 0.6 mV, and V1/2 = 12.9 ± 1.7 mV, k = 16 ± 1.4 mV for whole cell and inside-out whole cell, respectively.

DISCUSSION

Ion channels are attractive drug targets, as they are membrane proteins with a rich existing and potential pharmacology, with a well-developed set of “patch-clamp” techniques used to study them. These patch-clamp techniques can be used to characterize the actions of novel compounds on ion channels, but numerous factors limit using these techniques for compound screening. Important limitations include speed and ease of patch clamping, as well as hurdles facing parallel recording. Recently, significant efforts have been made by different research groups and biotechnology companies to automate patch-clamp recording, with variable success.2,6,10 Another often overlooked limitation, however, is the intracellular binding site of many ion channel pharmacological agents,15-17 which creates difficulties with identifying the structure-activity relationship of novel compounds due to the confounding variable of membrane permeability. In a step toward removing this limitation, we have developed a novel inside-out whole-cell recording method, in which the intracellular membrane is exposed to the bath solution. This configuration allows the movement of a recording electrode and cell through the air and into multiple compound-containing wells. This ability has allowed the automation of our recording process and will allow for the development of a parallel recording/compound application system.

The unique method for intracellular drug application used by this method allows multiple changes of intracellular saline within short time intervals. Moreover, because a high diffusion rate exists between the intracellular compartment and the bath saline, the same cell can be used for multiple drug applications. Potassium channel block by bath application of both 4-AP16,18 and E-403117,19 is known to be a very slow process in conventional whole-cell recordings, presumably due to their slow membrane diffusion and intracellular sites of action. In contrast, using this method of recording and drug application, a steady-state level of block was produced within 10 to 30 sec. Even more striking, in conventional whole-cell recordings, 4-AP washout is very slow and incomplete, whereas in this new system, the blocking effect was almost completely reversible within a very short (< 10 sec) time interval. Thus, these data support the role of slow membrane diffusion as a cause of the slow compound kinetics in conventional recordings with 4-AP and E-4031. When using the RoboPatch drug application method, the intracellular saline is changed simply by moving the patch pipette from one well to another because the cell membrane is protected from the atmosphere inside a fluid-filled glass capillary. Multiple-compound screening on a single cell is obtainable with this system, although like any system using this method, irreversibly acting compounds will necessitate the end of an experiment. Weakly active or reversible compounds may be detected by inserting control (wash) steps between compound testing wells.

One system that uses a similar recording configuration to that described here is the Flyion® system.2 In this technology, dissociated cells are loaded into a glass patch pipette, and cells are driven toward the tip via negative pressure applied at the tip end of the pipette, rather than at the back end of the pipette as in traditional recording. GOhm seals and disruption of the membrane to obtain a whole-cell configuration are again obtained using negative pressure at the pipette tip. Solutions are then applied to the extracellular surface of the cell by perfusing the pipette itself, rather than in the bath. This latter step has been described previously2 but has not been widely used due to its technical difficulty and slow equilibration time. In the system described here, we take advantage of the fact that (1) positive pressure applied internally to the pipette forces cells toward the tip and allows GOhm seal formation, and (2) upon seal formation, part of the cell is very close to, or protrudes from, the electrode tip. Thus, exposure to air will break this membrane piece without disrupting the cell-pipette seal, thus eliminating the need for suction on the outer side of the tip. In addition, our method extends the technique to applying agents intracellularly while still allowing perfusion of the pipette if access to the extracellular compartment is desired. The described method of inside-out whole-cell configuration allows multiple exposures to air, with no loss of seal viability. Thus, simple movements of the electrode tip to different wells in a microplate produce very fast drug application to the intracellular surface of the membrane and channel while simultaneously using a minimum of drug-containing solution.

An important issue determining the ease of obtaining GOhm seals in the new inside-out whole-cell configuration presented here is the quality of the patch pipette interior. By using high-quality, capillary-free glass electrodes, we routinely obtained a 70% to 80% success rate (true gigaseal and subsequent whole-cell formation) for numerous cell lines, including CHO-K1, U937, and HEK293. Similar to many of the automated systems, cells must be dissociated into single-cell suspensions prior to recording, using a method that does not damage the membrane proteins of interest. Combining the typical duration of recordings (> 30 min) and fast time of drug onset and washout, this should allow for the screening of approximately 45 compounds in a typical recording from a single cell, assuming that no irreversible or toxic compounds are included. The current success rate of ~70% would indicate that this
throughput should be sustainable throughout a typical recording session.

A number of technologies that have been developed recently may ultimately lead to a truly high-throughput ion channel screening platform. The RoboPatch system described here is clearly capable of recording from a single cell in an automated fashion. This could be expanded into a parallel system, as stable recordings can be obtained in parallel. GOhm seal formation generally occurs quickly in nearly all pipettes, and whole-cell formation is formed via exposure to air, thus indicating that a synchronous software system would be sufficient to control this process. Parallel, simultaneous recording from 8, 12, or more cells would be possible, opening the way for dramatically higher screening rates than currently exist with glass electrodes. Moreover, the inside-out whole-cell configuration could be used for creation of premanufactured arrays of biosensors. In preliminary experiments, we were able to perform freeze/thaw cycles of the cell/pipette preparations in inside-out whole-cell configuration without losing gigaseals. Providing a high success rate (>70%) of gigaseal formation, it could be possible to premanufacture inside-out membrane biosensors using simple pressure controller protocols and to keep these biosensors frozen for later use in electrophysiological experiments.

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