Population Patch Clamp Improves Data Consistency and Success Rates in the Measurement of Ionic Currents

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Present whole-cell patch-clamp methodology has only moderate consistency and throughput, rendering impractical functional measurements on large numbers of ion channel ligands or on large numbers of unknown or mutant channel genes. In the population patch clamp (PPC) described herein, a single voltage-clamp amplifier sums the whole-cell currents from multiple cells at once, each sealed to a separate aperture in a planar substrate well. The resulting ensemble currents are more consistent from well to well, and the success rate for each recording attempt is >95%. The PPC was implemented by modifying the PatchPlate substrate and amplifiers in the IonWorks patch-clamp instrument. The increased data consistency and likelihood of a successful recording in each well, combined with 384-well measurements in parallel, allow the direct electrophysiological recording of thousands of ensemble ionic currents per day. Therapeutic groups in drug discovery programs require this order of throughput to screen directed compound libraries against ion channel targets. The potential for studying the function of large numbers of ion channel mutants may be realized with the technique. The procedure incorporates subtraction methods that correct for expected distortions and also reliably produces data that agree with previous patch-clamp studies. (Journal of Biomolecular Screening 2006:488-496)

Key words: ion channel, electrophysiology, patch clamp, planar patch, population patch clamp

INTRODUCTION

VOLTAGE-CLAMP TECHNIQUES developed more than 50 years ago allowed the control of the membrane voltage and measurement of voltage-dependent ionic currents for the first time in large molluscan and amphibian cells.1-3 The patch-clamp technique, described in detail in 1981, is a refinement of voltage-clamp techniques that allows the measurement of currents in much smaller cells, including mammalian cells.4 Patch-clamp assays face a number of challenges, including modest success rates for each patch-clamped cell and the variability in cell-to-cell expression levels. Even in stably transfected cell lines, the measurable expression levels can vary 10-fold.5 These factors combine to force low throughput with conventional patch clamping of a single cell, vitiating 1) functional measurements on large numbers of compounds for studying the mode of drug binding or for drug discovery and 2) experiments on large numbers of unknown or mutant channel genes. Because of this, ion channels are a viable but underrepresented target class for drug discovery, whether in academic or pharmaceutical company contexts. In attempts to increase the throughput of patch clamping, automation was initially introduced by using robotics designed to mimic the human researcher or by positioning suspended cells at the surface of a drop of solution and approaching the cells with the recording electrode, but neither of these systems provided a substantial increase in throughput or data quality.6 A significant advance toward increased throughput was achieved when the pipette was replaced by a planar substrate containing an array of apertures, each 1 to 2 µm in diameter.7-11 Such automated systems have enabled a substantial increase in throughput and direct electrophysiological screening,12 but they have not changed the standard paradigm of 1 cell per measurement nor have they led to an improvement in data consistency. Success rates using planar patch techniques have been reported to range between 30% to 80% for obtaining a data point from an individual cell.9,12,13

We now improve data consistency and success rates by measuring the average response of many cells in parallel using the population patch-clamp (PPC) technique. PPC is performed by
modifying the existing substrate such that each recording well contains 64 recording sites (apertures) for cells instead of the usual single aperture. This allows for the recording of up to 64 cells in parallel instead of a single cell as done previously. Ionic currents on the modified substrate are measured using an IonWorks instrument with redesigned amplifiers to handle the larger currents. Such a change in the measurement paradigm is an extension of the whole-cell patch clamp itself. In whole-cell patch clamp, the measured current is the ensemble average of thousands of single-channel currents contributed by the individual ion channels distributed in the membrane. The PPC technique makes an ensemble measurement of the currents in cells sealed to an array of n measurement apertures (1 cell per aperture) in each well of a microplate using a single voltage-clamp amplifier per well. Ideally, the measured current is the average of the currents from all the voltage-clamped cells in 1 well, but in practice, it also contains confounding currents from apertures that did not successfully form a seal with a cell or from nonexpressing cells. Leak-subtraction techniques are used to identify and remove the confounding currents, and small residual effects of these currents are outweighed by the improvements achieved in data consistency and success rates using the PPC technique. The increase in data consistency that the PPC technique provides allows success rates for experimental runs using 384-well microplates to exceed 95%, and frequently runs are 100% successful.

MATERIALS AND METHODS

**Electrophysiology**

PPC measurements were performed using an IonWorks Quattro instrument (Molecular Devices Corporation, Union City, CA) using either PatchPlate™ PPC substrates (Molecular Devices Corporation) with 64 apertures per well or single-aperture PatchPlate substrates. The voltage-clamp amplifiers are capable of being switched to the appropriate gain for single-aperture or PPC substrates. Resistive (scaled) leak subtraction was used whereby the passive seal conductance is calculated at 2 hyperpolarized voltages and calculated as an ohmic conductance applicable to the entire voltage range used. The calculated leak current is digitally subtracted from the total current for each sample point acquired (however, the unsubtracted raw data are recorded). \(K_{1.3}\) currents were elicited by a voltage step from a holding potential of –70 mV to +40 mV for 300 ms, \(Na_{1.5}\) currents were elicited by a voltage step from a holding potential of –120 mV to –20 mV for 40 ms, human **ether-à-go-go-related gene** (hERG) currents were measured with a prepulse to +40 mV (5 s) followed by a step to –30 mV (4 s) to elicit the deactivating tail currents, and the holding potential was –70 mV. Compounds were incubated for 330 to 430 s to elicit the deactivating tail currents, and the holding potential pulse to +40 mV (5 s) followed by a step to –30 mV (4 s) to go-related gene

**PPC theory and practice**

The PPC technique is feasible because leak subtraction removes the linear leak current from the total measured currents to reveal the nonlinear ionic current of interest. With a single parallel measurement, the value of the seals in the 64 individual branches of the parallel circuit for each well is not known. Each individual branch consists of an aperture, the perforated cell and its membrane, and the leak conductance through the seal between the cell and substrate. In practice, the PPC technique works well when the PPC seal resistance averages ~50 MΩ or higher per aperture. We present a simulation at the end of the results section (Fig. 7) to address the numbers of completely open apertures or partially occluded apertures that can be present for the technique to still be successful.

**Cell lines and culture**

Chinese hamster lung (CHL) cells expressing \(Na_{1.5}\) channels, Chinese hamster ovary (CHO) cells expressing \(K_{1.3}\), or hERG channels were used. The hERG expressing line was a generous gift from Cytomyx (Cambridge, UK), the \(Na_{1.5}\) cell line was a generous gift from a Molecular Devices customer, and the \(K_{1.3}\) cell line was generated internally at Molecular Devices.

Cells were cultured in T-75 flasks and passaged every 2 to 3 days at 1:3 to 1:6 seeding density dilutions. Cells were also maintained at a lower seeding density (1:50) and passaged every 3 to 4 days. Flasks near confluence that were seeded at the lower seeding density cells were used frequently (approximately every 1-2 weeks) to provide the source of cells seeded at the higher density.

**Preparation of cells for electrophysiology**

Cells were grown to 70% to 90% confluence in a flask and removed from the incubator (37° C, 5% CO₂) 1 to 3 days after plating. Growth medium was aspirated from the culture flasks. Cells were gently rinsed with 2.5 mL Versene (Gibco Cat. #15050) to remove residual media. A total of 2.5 mL Versene solution was again added, and the flasks containing cells were incubated at 37° C. After 4 to 7 min, cells became visibly rounded and were easily dislodged from the bottom of the flask with a few brief taps on a solid surface. A total of 20 mL of phosphate-buffered saline was added to the flask, and the mixture was centrifuged at 800 rpm for 4 min. The cell supernatant was decanted, and the cell pellets were resuspended in 3 mL of
external solution followed by triturations using a 200-µL pipettor. The cell suspension was then added to the cell boat on the IonWorks Quattro instrument just prior to the experimental run. The cell suspension had a final concentration of ~2 to 3 million cells per milliliter; this corresponds to ~7000 to 10,000 cells added per well. The variability was due mostly to the difference in cell confluency in the flasks used.

Preparation of perforating solution

Amphotericin aliquots of 5.0 ± 0.3 mg (Sigma Cat. #A4888) were preweighed and stored at 4° C. Prior to cell preparation, 180 µL DMSO (Sigma Cat. #D-2650) were added to an aliquot of amphotericin. The amphotericin/DMSO solution was sonicated until soluble (~1 min), added to a 50 mL conical tube of internal buffer, and vortexed for ~1 min. The solution was stored in the dark until ready for use.

Data analysis

Concentration-response curves for tetracaine, lidocaine, 4-aminopyridine, and hERG channel inhibitors were fitted to a 4-parameter equation:

\[
\% \text{ of control} = 100 \left(1 + \left(\frac{[\text{drug}]}{IC_{50}}\right)^p\right)^{-1},
\]

where \( IC_{50} \) is the concentration of drug required to inhibit current by 50% and \( p \) is the Hill slope.

RESULTS

We describe the use of the PPC technique on 3 types of ion channels exogenously expressed in stable cell lines: Kv1.3 and hERG channels in CHO cells and Na\textsubscript{v}1.5 channels in CHL cells. A schematic of a single well and 2 adjacent wells in the microplate is shown in Figure 1. Cells are added to the well, and a vacuum is applied to the lower common chamber. Note that unlike single-aperture planar patch clamp, in PPC wells, there are multiple apertures per well. Figure 2 shows a typical recording of Kv1.3 currents. PPC ensemble average currents have been normalized in all figures to a single-aperture equivalent current by dividing the population total current by 64 (the number of apertures). At the time of the voltage step, the membrane capacitive transient current is seen followed by the characteristic slower-activating ionic-current response of the Kv1.3 channel. Leakage current through the seal is the time-independent current seen in the unsubtracted sweep of Figure 2A. The dashed line separates the time-dependent Kv1.3 currents (above the dashed line) from the pedestal leak current. A key to the success of the PPC technique is the fact that the leakage current is linear (ohmic) whereas the voltage-dependent ionic current is nonlinear with respect to voltage. The leak current is composed of 2 major components: the current around successfully patched cells and the current through apertures that are either open or partially occluded by debris. Resistive leak-subtraction techniques are applied to digitally subtract the calculated leak current at any voltage estimated from the response to a small hyperpolarizing test voltage step applied prior to test voltage steps. In the event the leak current changes slowly with time due to changes in the electrical seal, errors are minimized by implementing the leak-subtraction process at the start of each sweep. After subtraction of the leak current, the remaining current exhibits the characteristic activation time course of the Kv1.3 current recorded by conventional patch clamp (Fig. 2B).\textsuperscript{15} The ensemble averaging that is inherent to the PPC technique improves the signal-to-noise ratio, as can be seen by comparing the PPC recording of Figure 2C with the single-aperture recording of Figure 2D. The improved signal-to-noise ratio allows the measurement of small currents that cannot be resolved in the single-aperture mode.

Figure 3 illustrates the improved data consistency of the PPC technique. Typical current recordings from 8 adjacent wells measured using the single-aperture (Fig. 3A) and PPC (Fig. 3B) modes of recording are shown. The 8 wells displayed are representative of recordings taken from the two respective 384-well plates compared in this figure. The current amplitudes and time to peak for the PPC currents are much more consistent than the single-aperture mode.
The mentioned seal rate is not difficult to achieve. Cell optimization was sufficient density of cells is added to each well, the aforementioned seal resistance is greater than 50 MΩ, the technique generally fails. The advantage of the PPC technique over conventional or single-aperture planar patch clamp is the reduction of measurement variability due to nonuniform current expression and a substantial increase in the success rate of obtaining a data point from each attempted measurement. It is important to define success rate so that a comparison can be made between PPC and single-aperture substrates. Success rate is defined as the percentage of ionic current recordings obtained from a 384-well plate. A well can fail for any of the following 4 reasons: 1) The well can be unusable because the apertures are blocked by debris or a bubble, 2) the cells can fail to form a usable seal greater than 50 MΩ, 3) the overall current can be too small due to a lack of expression, or 4) the current amplitude can be unstable over time (±20% over 5 min). The graph in Figure 5 shows the success rates of PPC and single-aperture individual runs for K,1.3, Na,1.5, and hERG. The PPC technique is very predictable with optimized cells. Since optimizing the K,1.3, Na,1.5, and hERG cell lines, we have conducted more than 100 consecutive runs in which the PPC success rates were 95% to 100%.

PPC ensemble averaging reduces the measurement variability due to nonuniform expression, allowing for more consistent pharmacological results when testing compounds against ion channel targets. Thirty-two 10-point IC50 plots for 4-aminopyridine (4-AP) inhibition of K,1.3 channels are plotted in Figure 6A and were obtained using the PPC technique in a single run (66 min). The percentage of ionic current recordings is divided by 64 to allow currents to be represented as the average current per aperture in the chamber. Panel D is the measured current through a single aperture.

FIG. 2. (A) Unsubtracted total currents including K,1.3 and leak currents measured with a single amplifier per well. Activation of the voltage-dependent current is seen after the decay of the capacitive transient; the ionic current is separated from the leak current by the dashed line. The dotted line is the baseline current at the holding potential. (B) Voltage-dependent current after passive leak current has been subtracted. (C, D) Human ether-à-go-go-related gene (hERG) potassium channel current measured using the population patch clamp (PPC) (C) and the single-aperture technique (D). Note the greater signal-to-noise ratio in the PPC trace. In panels A through C, the displayed current is the measured current divided by 64 to allow currents to be represented as the average current per aperture in the chamber. Panel D is the measured current through a single aperture.
Eight hERG inhibitors were also tested and the following IC_{50} values were obtained (in µM): astemizole 0.024, cisapride 0.051, dofetilide 0.016, flunarizine 1.8, imipramine 8.3, pimozide 0.090, terfenadine 0.439, and quinidine 1.7. These values are similar to those reported in the literature.

Two simulations to describe the PPC seal resistances

Because a single parallel measurement is made in a PPC well from multiple recording sites and multiple cells, there is no way to determine what the values of the seals are in the individual wells.
branches of the parallel circuit. For this reason, we have provided 2 simulations (Fig. 7) to help estimate the proportion of "successful" seals, partially occluded apertures, and completely open apertures within the parallel circuit. The 1st simulation assumes that there are only 2 populations of seals at the recording sites, one being successful seals (120 MΩ) and the other completely open apertures (3 MΩ). As the seal rate drops from 100%, the mean seal resistance for the parallel circuit drops off sharply. The measured range in seals that we typically see (50-120 MΩ) corresponds to less than 3% of the apertures being completely open (1 or 2 of 64 apertures). The 2nd simulation also assumes 2 populations of seals at the recording sites, one being successful seals (120 MΩ) as in the 1st simulation and the other population being partially occluded apertures assumed to be 10 MΩ. In this simulation, the measured range in seals that we typically see corresponds to less than 12% of the apertures (up to 7 of 64) being partially occluded. The actual situation is probably something in between these 2 simulations. In other words, there is probably a mixture of successful seals, partially occluded apertures, and completely open apertures. In any case, both simulations suggest that the seal rates within a single well must be 88% or greater. Once a cell line is optimized, this is not difficult to achieve on a routine basis.

DISCUSSION

The direct measurement of ion channel currents in drug discovery settings has been limited to the measurement of individual cells using conventional pipette or single-aperture planar patch clamp. The problems associated with these techniques include the loss of many data points due to data inconsistencies caused by the variability in channel expression levels, failed measurement attempts caused by technical problems including low seal resistances, and failure to obtain the whole-cell recording

FIG. 4. Current amplitude histograms for individual wells using a single-aperture versus 64-aperture (population patch clamp; PPC) substrate. The PPC histograms (blue) have a narrower distribution when compared to histograms constructed from single-aperture single-cell recordings (brown). The coefficient of variation (CV) is 2- to 4-fold smaller on the PPC histograms. Nonexpressing cells comprise the distribution of cells on the single-aperture histograms near zero current magnitude. The single-aperture Gaussian fits exclude the nonexpressing cells, whereas summary statistics include all cells including the nonexpressing cells. hERG = human ether-a-go-go-related gene.

FIG. 5. Success rates for individual wells in the single-aperture and population patch clamp (PPC) recording modes. Bar graph showing a 99.3% rate (n = 3456 wells) for Kv1.3 channels in PPC mode compared to 80.1% (n = 4608 wells) for the single-aperture mode. The success rate for Na, 1.5 channels in PPC mode was 95.5% (n = 3072 wells) and 71.3% (n = 3456 wells) in single-aperture mode. Human ether-a-go-go-related gene (hERG) channels had success rates of 97.3% for PPC mode (n = 1536 wells) and 61.0% for single-aperture mode (n = 3072 wells). Experimental runs are shown that were designed specifically to measure current stability, whereby saline (mock compound) is added instead of compound containing solution across the entire microplate.
configuration. Drug discovery programs need the capability to screen tens of thousands of compounds over a period of a few weeks with a low percentage of lost compounds so that a retesting of large numbers of compounds is not required. PPC resolves many of these issues, including data inconsistencies caused by a lack of channel expression and data points lost due to low seals. Ensemble averaging of the currents from 64 cells results in data points rarely being lost due to nonexpressing cells. The key to this success is that it does not matter that a small percentage of the patch-clamped cells in each well do not express exogenous currents; what matters is that a substantial number of them do indeed express currents of interest and that these currents contribute to the measured ensemble average current. All small endogenous currents present in nonexpressing cells are overwhelmed by the overall expressed current and essentially averaged out of the final current signal. Poor seals (<50 MΩ) that occur in the planar patch single-aperture mode are indeed present in the PPC mode as well; these relatively small linear currents are averaged into the larger total measured current and then subtracted out to yield the large nonlinear ensemble currents of interest.

**FIG. 6.** Pharmacology of K\(_{1.3}\) and Na\(_{1.5}\) channels. (A) Thirty-two 10-point IC\(_{50}\) curves for 4-amino pyridine (4AP) on K\(_{1.3}\) channels obtained using the population patch clamp technique in a single run (66 min) using a 384-well compound plate. (B) Sixteen IC\(_{50}\) curves each for tetracaine and lidocaine were obtained in a single run (70 min). CV = coefficient of variation.

**FIG. 7.** Prediction of percentage of open apertures assuming the seal resistance for properly clamped cells is 120 MΩ and all other apertures are partially occluded at 10 MΩ or completely open at 3 MΩ. The simulation predicts that the number of open apertures (at 3 MΩ) does not exceed 3% and the number of open apertures (at 10 MΩ) does not exceed 12%. PPC = population patch clamp.
The single-aperture planar patch technique using the same substrate used here\textsuperscript{9,12} was initially greeted with skepticism by some electrophysiologists because of the fact that the technique uses sub-G\(\Omega\) seals in the hundreds of M\(\Omega\) range. Questions were raised about the signal-to-noise ratios, the minimum current size that could be measured, and the ability to do biophysical studies with sub-G\(\Omega\) seals. We acknowledge that sub-G\(\Omega\) seals are not suitable for the study of subtle biophysical changes in the kinetics of ionic currents, particularly because the present-generation instruments do not incorporate any kind of dynamic series resistance correction. However, the signal-to-noise ratios and therefore the minimum currents that can be measured are acceptable for screening, and the single-aperture technique has proven to be robust as a screening tool.\textsuperscript{9,12} PPC uses seals of similar magnitude; however, the increased data consistency and improved signal-to-noise ratio may allow the detection of smaller currents including endogenous currents, but this remains to be seen. The measurement of ionic currents using sub-G\(\Omega\) seals is not meant as a replacement to conventional patch recording but as a much higher throughput form of screening using a direct electrophysiological assay.

**Compound and mutant screening**

The consistency of the PPC technique is a clear advantage in compound screening because it makes it unnecessary to use replicate compound applications to be confident of screening the majority of compounds in the library. The consistency is also an advantage for detailed studies on the pharmacology of individual compounds. For example, because of the inherent consistency in the PPC method, it is possible to obtain 10-point dose-response curves from 10 wells, obviating the need to average many responses from many wells at each concentration to reduce the variability at each concentration to an acceptable level.

The screening of large numbers of mutant channels can also be envisioned using the PPC technique in both academic and drug discovery settings. Clonal selection is already routinely performed\textsuperscript{39} using single-aperture planar patch-clamp format, whereby 12 individual clones of the same channel can be tested with an \(n\) of 32 for each clone in a 384-well run in 1 hour. Because of the consistency of PPC currents, kinetic studies could be carried out on transiently transfected ion channel mutants, with up to 48 different mutants tested in a 1-h run, with an \(n\) of 8 PPC wells per mutant. An \(n\) of 8 PPC wells should be sufficient to reliably detect subtle kinetic changes, as the ensemble current in each well is already inherently averaged from up to 64 individual cells. Ion channel studies in which potential regulatory sites (e.g., phosphorylation, calcium binding, voltage-sensing regions) have been knocked out by site-directed mutagenesis could be tested, and the effect of these mutations could be examined related to current kinetics, regulation, or expression. Whole regions of the ion channel proteins could be explored, or all the consensus sequences for a particular regulatory molecule could be individually mutated and scanned for effects very rapidly using PPC.

Larger scale genetic rescue screening could also be performed on nonconducting channels in which a mutation has caused a loss of function. Frequently, interaction pairs between amino acid residues or a particular tertiary structure within a channel protein are required for proper channel function. A mutation scan substituting all amino acids at a particular residue can be performed in the suspected area of residue interaction or tertiary structure disruption. Because the nonconducting mutants will not contribute any current to the ensemble average, multiple mutations could be pooled and tested in a single PPC well. Multiplexing the mutant screening in this manner would increase the rate of mutants screened; the degree of multiplexing at the single-well level would be determined by the level of desired sensitivity of the rescue assay. Increasing the number of mutants pooled into a single well would inherently reduce the output signal from any given rescue mutant due to the averaging of signals from the other mutants. Thus, the threshold of detection of the assay could be titrated: Pooling many clones into a PPC well would reduce the sensitivity but increase the rate of the screen, whereas reducing the number of clones pooled would increase the sensitivity of the assay.

The PPC is a robust technique that delivers more consistent and reproducible results than conventional whole-cell patch clamp with glass pipettes or single-aperture planar patch clamp. Consistency in each measurement is achieved by using the ensemble average of a large population of whole-cell currents. The main difference between the PPC technique and recording individual whole-cell currents from 64 wells is that in the PPC technique, the averaging is an intrinsic process done in the analog domain. Whole-cell currents from a population of cells converge in the well into a single analog amplifier, whereas in the alternative, the averaging is a digital process that requires currents from 64 wells to be separately acquired, then averaged. The consistency of the measurements from each well combined with the measurement of 384 wells boosts the throughput of the PPC technique to a level that could never be achieved with conventional electrode or single-aperture planar patch clamp.

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