Chapter 10

Automated Patch Clamping Using the QPatch

Kenneth A. Jones, Nicoletta Garbati, Hong Zhang, and Charles H. Large

Abstract

Whole-cell voltage clamp electrophysiology using glass patch pipettes (1) is regarded as the gold standard for measurement of compound activity on ion channels. Despite the high quality of the data generated by this method, in its traditional format, patch clamping has limited use in drug screening due to very low throughput. Over the years, developments in microfabrication have driven the development of planar, multi-aperture technologies that are suitable for parallel, automated patch recording techniques. Here we present detailed methods for two common applications of the planar patch technology using one of the commercially available instruments. The results demonstrate (a) the high quality of whole-cell recordings obtainable from cell lines expressing human $Na_v1.2$ or hERG ion channels, (b) the advantages of the methodology for increasing throughput, and (c) examples of how these assays support ion channel drug discovery.

Keywords: Automated patch clamp, Electrophysiology, QPatch, $Na_v1.2$, hERG, Ion channels.

1. Introduction

Whole-cell voltage clamp using patch electrodes *(1)* is regarded as the gold standard for measuring the physiological and pharmacological properties of ligand-gated and voltage-gated ion channels. Through an electrode attached to the cell membrane, current generated by ions flowing through ion channels in the cell membrane can be measured while the membrane potential is clamped to defined voltages. Despite the high-quality data generated by this method, throughput is relatively slow and not suitable for testing hundreds of compounds or many compounds at multiple concentrations. While ion channels traditionally have made excellent drug targets, lack of parallel recording methods has caused a bottleneck in screening for this important class of transmembrane proteins.

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In traditional patch clamping, glass electrodes are manually fabricated using a heated electrode puller and used to record from cells one at a time. In the 1990s, investigators began developing planar materials featuring 1–2 μM diameter holes suitable for obtaining high-resistance seals against cells *(2)*. After many refinements, glass- or silicon-based chips containing multiple apertures were designed to be used with sophisticated hardware and electronics such that cell positioning, gigaseal and whole-cell formation are obtained automatically. Several such devices including the PatchXpress® *(3, 4)*, developed by Axon Instruments; QPatch® *(5)*, developed by Sophion; and IonWorks® *(6)*, distributed by Molecular Devices, have now been successfully introduced into the market and are being used by pharmaceutical, contract and academic research labs.

At the heart of the QPatch is a disposable chip that contains an array of 16 micro-fabricated holes enabling voltage clamp measurements to be made in parallel. A carefully prepared cell suspension is added to the chip units and negative pressure applied to attract a cell. Increasing this pressure allows the formation of a gigaseal, and subsequent pressure ramps rupture the cell membrane providing a whole-cell configuration. Once a whole-cell configuration has been achieved, voltage protocols are executed, and the system then adds drugs using a single or multiple robotic pipettor. Data are recorded and stored on a dedicated server.

Many CNS-active drugs have a tendency to cause unwanted effects on ion channels responsible for electrical conduction in the heart. A potassium channel, human *ether-a-go-go-related gene* (hERG) *(7, 8)*, critically responsible for the repolarising phase of ventricular action potentials, is associated with adverse events such as QT interval prolongation when blocked by drugs *(9)*. To a great extent, the impetus for the commercial development of automated patch clamping arises from the need of the pharmaceutical industry to develop drugs that do not inhibit hERG at therapeutic concentrations and thus have an improved cardiovascular safety profile.

Voltage-gated sodium channels (VGSC) have a fundamental role in most electrically excitable cells since they conduct the inward currents that occur during the rising phase of action potentials. The main pore-forming component of these channels is the α-subunit, of which nine different subtypes have been identified *(11)*. The human subtype $Na_v1.2$ is predominantly expressed on the axons of central neurones *(11, 12)*. The channel may exist in at least three different conformational states, resting (closed), open or inactivated, and the transition between these states is voltage dependent *(13)*. The mechanism of activation of this ion channel is membrane depolarisation, which causes a conformational change in the protein (open state) allowing Na^+ ions to permeate the channel. Channel opening is followed by a rapid (<2 ms) transition to an inactivated state. Upon repolarisation of the cell

membrane, channels recover from this inactivated state and transition back to the closed or resting state. Drugs that stabilise the inactivated state of voltage-gated sodium channels have an established role in the treatment of neurological and some psychiatric disorders *(14)*. Given the voltage- and time-dependent nature of drug interactions with the inactivated state of sodium channels, estimation of the affinity of drugs for this state requires a functional assay such as patch-clamp electrophysiology *(15)* and cannot be achieved using other currently available high-throughput screening techniques, such as voltage-sensitive dyes or radioligand binding. Consequently, the advent of planar patch technology has opened up the possibility for effective screening against this target and the identification of novel sodium channel blockers with improved selectivity.

This chapter describes the application of the 16-channel QPatch (Sophion Bioscience A/S) to estimate the affinity of novel compounds for the inactivated state of $Na_v1.2$ and to determine the potency of compounds blocking hERG.

2. Materials

2.1. Cell Culture and Cell Preparation

1. Dulbecco's modified Eagle's Medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and geneticin (Gibco) as the selection antibiotic (400 μg/mL).
2. 293 SFM II medium (Gibco) containing 25 mM HEPES (Gibco) and 0.04 mg/mL soy bean trypsin inhibitor (Sigma) (*see* **Note 2**).
3. D-PBS (Dulbecco's w/o: calcium, magnesium, w/o sodium bicarbonate GibcoBRL, Invitrogen).
4. Versene (Gibco).
5. Trypsin/EDTA (T/E) (10 ×) (Gibco, Life Technologies).
6. DMEM/F12 + Glutamax™ cell culture medium (Gibco) supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin (Invitrogen) and 500 μg/mL G418.
7. Detatchin™ (Genlantis).

2.2. Electrophysiological Solutions to Record $Na_v1.2$ Currents

1. Internal solution: CF 140 mM, EGTA 1 mM , NaCl 10 mM, HEPES 10 mM, pH 7.3 with CsOH (need ~500 μL 2.5 M CsOH in 1000 mL). Note: adjust osmolarity to 320 mOsm with sucrose, CsF will precipitate at 4°, EGTA must be dissolved in CsOH (380 mg EGTA in 5 mL 0.75 M CsOH). Filter the solution.

2. External solution: NaCl 140 mM, KCl 3 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1 mM , Glucose 11 mM, HEPES 10 mM, $CdCl_2$ 0.1 mM, TEA Cl 20 mM, pH 7.3 with NaOH. Adjust osmolarity to 320 mOsm with sucrose. Filter the solution.

2.3. Electrophysiological Solutions to Record hERG Currents

1. Internal solution: KCl 100 mM, KF 30 mM, EGTA 10 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, HEPES 10 mM, K_2ATP 5 mM, Adjust pH 7.2 with KOH, adjust osmolarity to 305 with glucose (*see* **Note 1**).
2. External solution: NaCl 140 mM, KCl 4 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM, Glucose10 mM, adjust pH to 7.4 with NaOH, adjust osmolarity to 295 with glucose.

2.4. Drugs

Test compounds are dissolved in DMSO at a concentration of 10–100 mM. After dilution in extracellular solution, final DMSO concentration is 0.1% (or less) and has no obvious effects on sodium or potassium currents (*see* **Note 3**).

3. Methods

A general procedure for planar patch clamping can be applied to all studies of ion channels. Steps include the following: (1) preparation of a high-density suspension of cells in external solution (3–5 $\times$ 10^6 cells per mL – typically 1 T75 culture flask), (2) preparation of protocols on the instrument including the protocol for achieving whole-cell mode, voltage protocol and the drug application protocol and (3) preparation of drug solutions. A separate drug plate, typically 96 well, contains solutions to be used either in single-point screening mode or in a multi-point, concentration–effect mode (*see* **Note 4**). Once the cell suspension, drug plate and patch plate are ready and in position on the instrument platform, the experiment can begin under full robotic control. Analysis of data can be done using either built-in software routines or third-party methods.

3.1. Cell Culture

HEK293 cells stably transfected with cDNA encoding the human brain type IIa sodium channel α-subunit (GSK R&D, UK) were grown in DMEM supplemented with 10% heat-inactivated foetal calf serum and geneticin as the selection antibiotic (400 μg/mL).

CHO cells stably expressing human hERG were purchased from AVIVA Bioscience *(4)* and grown in DMEM/F12 medium supplemented with 10% FBS, 1% pen/strep and 1% l-glutamine. Cells were grown in standard, vented 75-cm^2 flasks in a 5% CO_2 atmosphere at 37°C.

3.2. Sub-culturing Cells

Cells are passaged using standard methods with care taken to split cells when confluency reaches 70% for Nav1.2 cells and 80% for hERG cells. Permitting overgrowth at any time during passaging can result in a dramatic loss of expression. Both cell types are enzymatically dispersed to ensure uniform seeding onto daughter plates.

3.3. Preparing Cells for Experiments

An example of the weekly cycle of cell culture for HEK293 cells is provided (**Table 10.1**): On Monday, two or more T75 flasks (80% confluent) are split; three T75 flasks are plated at 1×10^6 cells/mL for use on Wednesday and three T75 flasks are plated at 5×10^5 cells/mL for use on Thursday. On Wednesday it is also necessary to repeat the split and plate at 1×10^6 cells/mL to provide sufficient flasks for further experiments on Friday and to get mother flasks for the next cycle.

Table 10.1
Example of a weekly cycle of cell culture to provide cells for experiment on 3 days

Monday	Tuesday	Wednesday	Thursday	Friday
Two mother flasks		Three T75 flasks (1×10^6)	Three T75 flasks (5×10^5)	Three T75 flasks (1×10^6)
		Two mother flasks		Two mother flasks

It is important that cells in the T75 flasks for use in the assay are ~70% confluent. Cell isolation into a single-cell suspension for addition to the QPatch is aided if the cells are thinly spread out rather than in rafts forming a monolayer (*see* **Note 5**).

3.4. Harvesting and Preparation of Cells Using the QPatch On-Board Stirrer

1. Remove growth medium from the culture flask by aspiration when cells are ~70% confluent.
2. Wash cells 1 × with D-PBS and remove buffer. Add ~6–7 mL T/E 1 × (ensure an even distribution of T/E), then remove the T/E and leave the culture flask to rest for ~1 minute in the incubator. Ensure that cells are rounded up before proceeding. If not, continue incubation.
3. Detach cells by firmly tapping the sides of the flask until the cells loosen from the bottom. Add 10 mL of fresh 293 SFM II medium containing 25 mM HEPES and 0.04 mg/mL soy bean trypsin inhibitor to the first of the three flasks and resuspend the cells by gently working the cell suspension up and down a pipette 5–10 times to break up cell clumps.
4. Immediately add the cell suspension to the storage container on the QPatch and start stirring.

3.5. Recording from Na Channels: Validation of the QPatch hNaV1.2 Assay

3.5.1. Biophysical Characteristics

The basic biophysical characteristics of sodium channels can be studied by the application of a family of voltage steps. Cells are held at a potential of –90 mV. Depolarizing voltage steps of 10 ms duration are applied to a range of potentials (–40 mV to +40 mV, in intervals of 10 mV) (**Fig. 10.1a**). By measuring the current that is generated at each step potential (**Fig. 10.1b**) and applying leak subtraction (P4), a current–voltage plot can be generated using the QPatch software (**Fig. 10.1c**). In studies conducted with the HEK293–hNaV1.2 cell line, the maximum peak inward current was evoked with a test pulse to –10 mV. Furthermore, extrapolation of the curve suggests that the inward current reverses at around +50 to +60 mV, consistent with the reversal potential of sodium ions observed in manual patch clamp experiments under similar conditions.

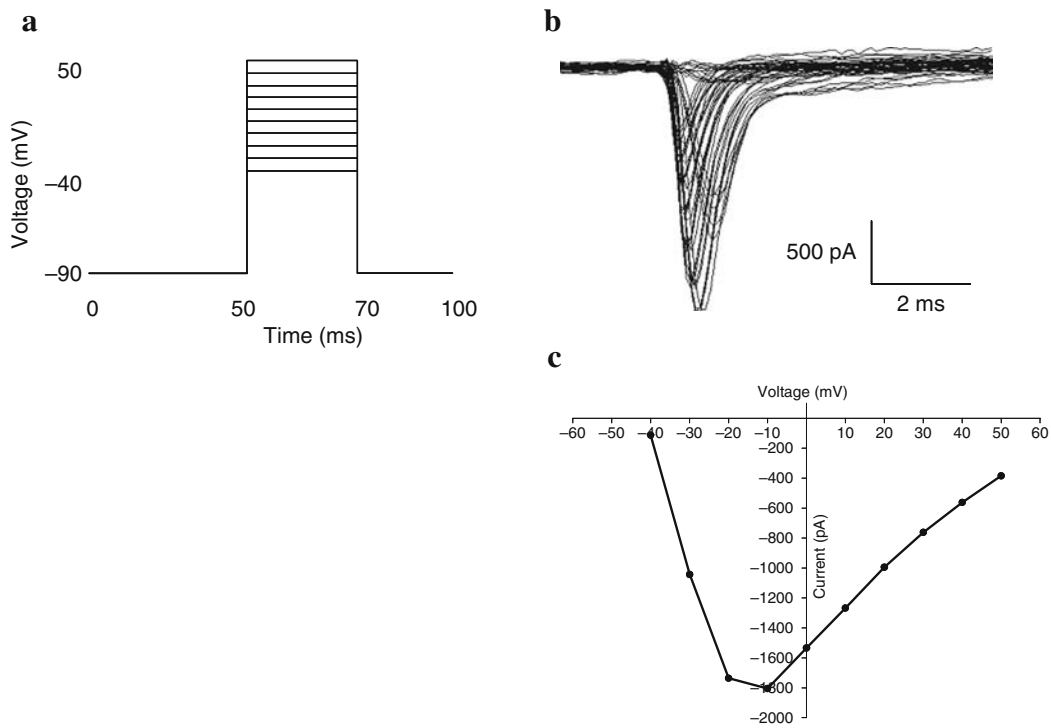

Fig. 10.1. (**a**) Voltage protocol used to construct an I–V plot from HEK293–hNaV1.2 cells. Cells were held at a membrane potential of –90 mV and stepped to a range of potentials (–40 to +40 mV) for 10 ms. (**b**) Examples of the current responses recorded in response to the family of voltage steps. (**c**) An I–V plot of these data shows that the peak sodium current could be evoked with a voltage step to –10 mV, consistent with results from similar experiments using manual patch-clamp electrophysiology.

3.5.2. Sensitivity to DMSO

DMSO is typically used to dissolve test compounds. At high concentrations the solvent can disrupt plasma membrane properties and may affect the quality of the recordings and pharmacological

results. Consequently, the sensitivity of the QPatch hNaV1.2 assay to DMSO was determined by applying the voltage protocol used to construct an I–V plot in the presence of increasing concentrations of the solvent (data not shown). Results suggest that a maximum concentration of 0.3% v/v should be used, since higher concentrations adversely affected current responses. In practice, concentrations of 0.01–0.1% v/v DMSO are generally sufficient to ensure the solution of most compounds up to concentrations of 10 μM in physiological saline.

3.5.3. Stability of Recordings Over Time

The longevity of whole-cell recordings is important since the protocol used to estimate the affinity of a compound for the inactivated state of the $Na_V1.2$ channels is quite long with respect to the simple I–V determination discussed earlier. As a result, it is important that stable recordings be maintained for 20–30 minutes. Therefore, in a further set of experiments the viability of whole-cell recordings from HEK293–NaV1.2 cells was followed over time. For these experiments, 20 ms test pulses to 0 mV were applied from different holding potentials at an interval of 7 s. Analysis of the peak current evoked by each pulse over time (**Fig. 10.2**) shows that viable recordings could be maintained for at least 20 minutes when cells were held at relatively hyperpolarised potentials of –90 or –120 mV. At a more depolarised holding potential of –70 mV, there was an evident rundown of the inward current, possibly due to gradual accumulation of the channels in the inactivated state.

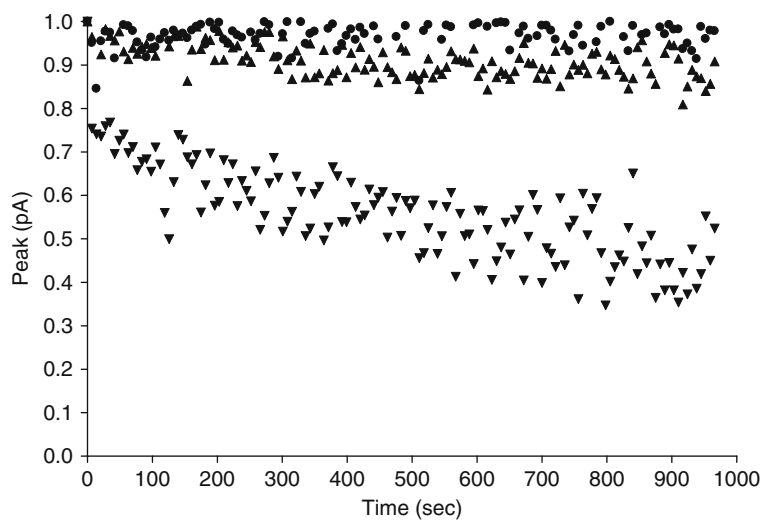

Fig. 10.2. The time course of inward sodium currents evoked by voltage steps to 0 mV at intervals of 7 s. Data shown are the mean peak amplitudes of the current responses from cells held at –120 (●), –90 (▲) or –70 (▼) mV ($n = 7$).

3.6. Criteria for Excluding Cells from the Analysis

It was possible on occasion to obtain 14–15 stable whole-cell recordings from each 16-well QPlate, although the average over a 10-plate trial run was 8.5 (or 53%) (**Table 10.2**). Cells were included in the analysis, however, only if they met additional requirements: formation of seal with a resistance of >0.1 MΩ; in whole-cell configuration, the series resistance remains <10 MΩ for the duration of the experiment; the peak current evoked from a conditioning pulse of –120 mV is greater than ~200 pA.

Table 10.2
Success rates defined by various criteria for HEK293–NaV1.2 cell line

Number of plates tested	Whole-cell rate (%)	Completed experiments (%)	Cells used for analysis (%)
10	53	49	39

Average per plote (out of 16 possible).

3.7. Estimation of the Affinity of Compounds for the Inactivated State of hNaV1.2 Channels

3.7.1. Voltage Protocol to Investigate Steady-State Inactivation

A protocol to study steady-state inactivation of the hNaV1.2 channels was developed based on one described by Bean et al. and Kuo and Bean *(15, 16)*. The HEK293–hNaV1.2 cells were held at –120 mV and stepped to a range of conditioning voltages (–120 to –40 mV) for 9 s to induce steady-state inactivation. At the end of each conditioning period, the cell was stepped to +20 mV for 2 ms to elicit residual sodium currents.

3.7.2. Data Analysis

For each cell, the peak current is plotted against the conditioning voltage (**Fig. 10.3**). This plot illustrates the voltage dependence of inactivation of the channels and can be fitted to the following Boltzmann equation:

$$Y = 1/1(1 + \exp((V - Vh)/(V - Vh)k)).$$

where Vh represents the voltage at which inactivation reaches midpoint and k is the slope factor.

The shift in the Vh value (ΔV) is determined for each drug concentration tested. The values of ΔV thus obtained are then normalised with respect to the slope factor and are used to estimate the Ki using the following equation:

$$\mathrm{Exp}\,(\Delta V/k) = (1 + (X/))/(1 + (X/Ki))(1 + (X/KR))$$

where X is the drug concentration, KR is the affinity constant for the resting state of the channels [KR is approximately equivalent to the IC_{50} value obtained from the concentration–response curve at a holding potential of –110 mV, where no inactivation is present *(17)*].

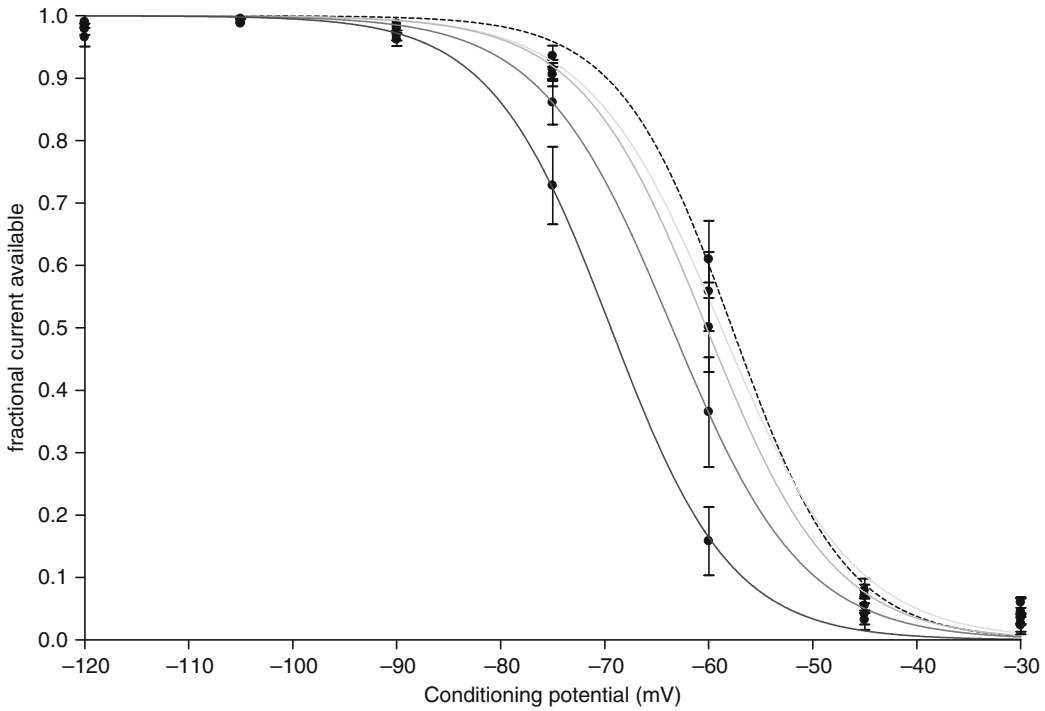

Fig. 10.3. Current–voltage plots showing the inactivation of sodium currents with more depolarised conditioning potentials. Data shown are the mean ± SEM ($n = 9$ cells) of the peak currents at each conditioning voltage fitted to a Boltzmann equation. Drug application causes a concentration-dependent, leftward shift of the curve. *Dotted line* – Control; *solid line* – increasing concentration of drug.

A comparison of Ki values determined using QPatch and manual patch electrophysiology for a series of sodium channel inhibitors is shown in **Fig. 10.4**; there is good agreement with values obtained using manual patch clamp performed under similar conditions and with a similar voltage protocol.

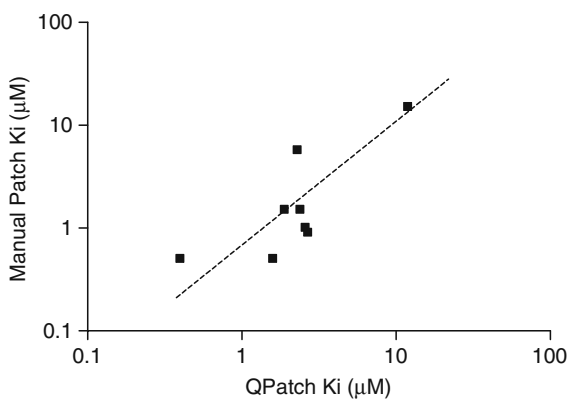

Fig. 10.4. The estimated affinity (Ki) of a series of sodium channel blockers for the inactivated state of human NaV1.2 channels expressed in HEK293 cells. Data shown were determined from at least five cells using QPatch and conventional manual patch-clamp techniques. Voltage protocols and other experimental conditions were similar between the two techniques.

3.8. Recording Whole-Cell hERG Currents

3.8.1. Preparing the Cell Suspension

Flasks are selected at 50–90% confluency, media are removed, cells are washed once with D-PBS and 4 mL of Detachin™ is placed evenly around the cells. The flask is returned to the incubator for 7 minutes. Subsequently the flask is observed on the microscope (10× objective) for the presence of rounded and freely floating cells. Tapping of the flask is not necessary. Cells are gently suspended by pipetting the solution up and down along the bottom of the tipped flask three to four times. The cell suspension is removed and placed in a 15-mL conical tube and centrifuged at 1000× rpm for 2 minutes. The supernatant is removed; cells are resuspended in 5–7 mL of external solution and centrifuged again at 1000 rpm for 2 minutes. The supernatant is removed and 300–500 μL of external solution is added to achieve a final cell density of about 3×10^6 cells/mL (final volume depends on the number of cells in the sample).

3.8.2. Drug Plate Preparation

Stock solutions of compounds in DMSO at 10 mM are diluted to 10 and 30 μM in EC solution; the 10 μM solutions are further diluted to make 3 and 0.3 μM concentrations. These are placed in 96 deep well plates (1 mL volumes) with each compound occupying one partial row. In the job set-up, the minimum and maximum compound repetition variables determine the number of replicates for each individual compound. This method obviates the need to repeat rows for each compound, simplifies drug plate preparation and provides more flexibility when repeated jobs are required to provide the required number of replicates.

3.8.3. Voltage, Whole-Cell and Application Protocols

The protocol for obtaining gigaseals and whole-cell configuration is a standard protocol supplied by Sophion Bioscience for CHO cells. After whole-cell mode is obtained, cells are held at –90 mV. hERG currents are activated by stepping to +20 mV for 2 s and then to –50 mV for 4 s (**Fig. 10.5**). This second repolarizing step generates an outward tail current resulting from the recovery from inactivation *(18)* (**Fig. 10.6**). The pulse protocol is repeated every 12 s until the last drug concentration is tested (about 25 minutes). Typically several additions of external solution are made while the baseline is being established. Subsequently, each drug concentration is added two or more times to ensure a complete exchange of the new solution.

3.8.4. Criteria for Selecting Cells for Analysis

Typically 14–15 stable whole-cell recordings are obtained from each Qplate (**Table 10.3**). Of these, one to two may be rejected based on low current (<100 pA) or current "run-down", defined as a progressive loss of current over time.

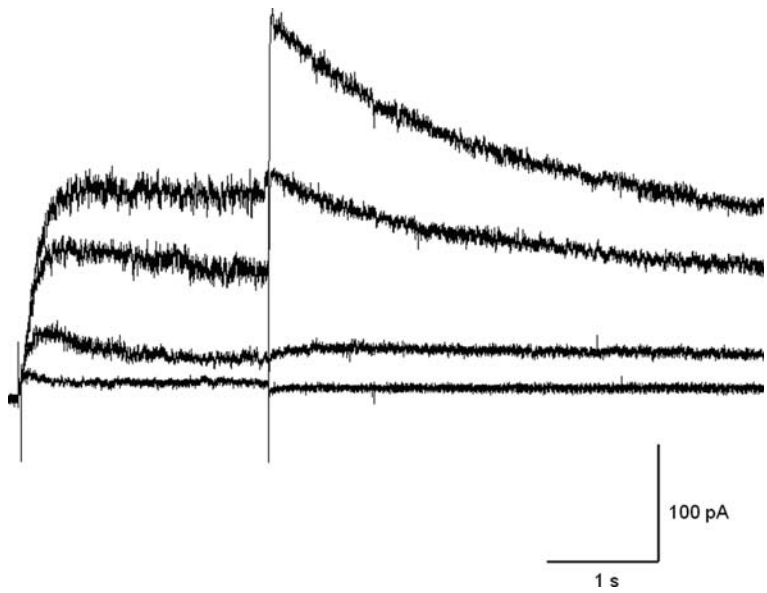

Fig. 10.5. hERG outward tail currents elicited from a voltage-clamped cell on the QPlate. *Arrows* mark the times where resting currents and peak tail currents are measured. Note the progressive loss of current as haloperidol is applied at concentrations ranging from 30 nM to 10 μM.

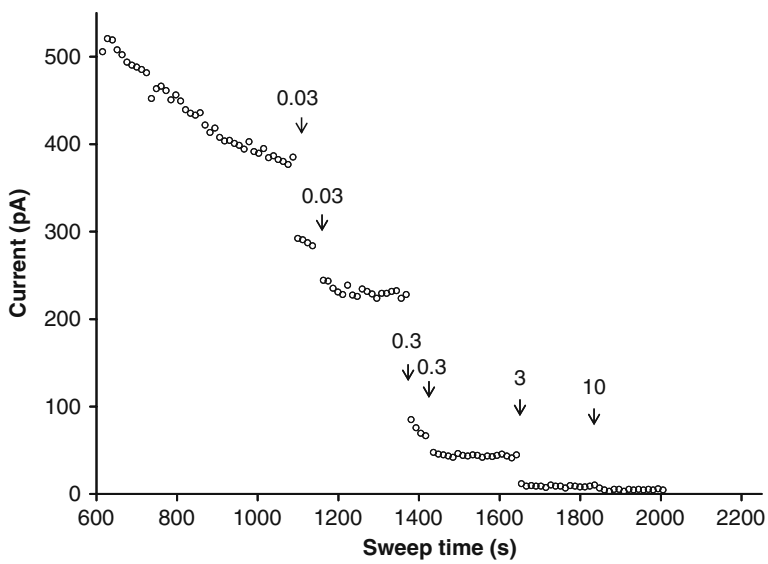

Fig. 10.6. Plot of peak tail currents (minus resting current) from example cell shown in **Fig. 10.5**. Note the modest rundown that occurs during the first 400 s and subsequently stabilises. Drugs are applied (*arrows*) twice for the first two concentrations and once for the last two concentrations. Note also that the first of the two drug additions do not produce a steady-state inhibition.

Table 10.3
Success rates defined by various criteria for CHO–hERG cell line

No. plates tested	No. cells tested	WC rate*	Completed protocols*	Used for data analysis*	No. current rate*	Run-down rate*
8	114	89	74	53	2.6	17

*Expressed as percent of total number of cells tested.

Cells are rejected if rundown exceeds 5% per minute or if more than 25% of current is lost before the first drug addition occurs.

3.8.5. Data Analysis

IC_{50}s are determined using the QPatch analysis software. Peak outward current at –50 mV, minus the holding current measured at a 5-ms pre-pulse to –50 mV, is plotted every 10 s. The stability of the current over time and the effect of drugs are inspected; data are excluded from cells if they exhibit excessive rundown, have currents less than 80 pA or if drug effects do not plateau (**Fig. 10.7**). Sigmoidal curve fits are calculated using the QPatch analysis software using the last buffer response to define the 100% current value (**Fig. 10.7**). **Table 10.4** shows IC_{50} values for reference compounds compared to literature values. In general, there is a good agreement compared to manual patch-clamp methods.

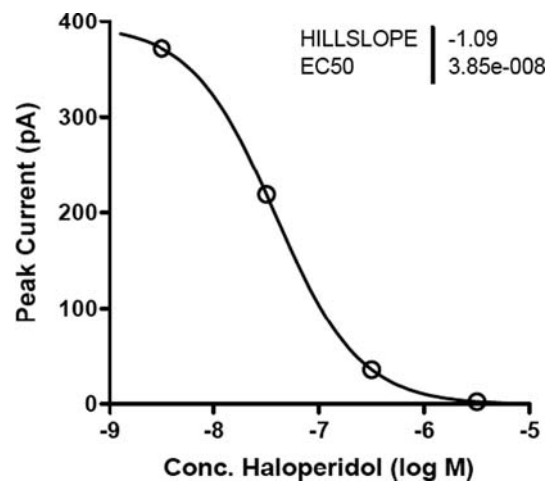

Fig. 10.7. Example concentration–effect response for haloperidol. Peak current is determined for each concentration of compound, using the second addition for double addition protocols, and the curve fit is generated using the Hill equation.

Table 10.4
Average IC_{50} values for reference compounds at hERG determined using the QPatch compared to manual patch-clamp methods

	QPatch (nM)[a]	Manual patch clamp (nM)
Haloperidol	36	15[b]
Amiodarone	1374	48[c]–1000[d]
Quinidine	681	300[d]
Clozapine	1510	1700[b]
Cisapride	30	15[c]
Aspirin	>30,000	>30,000[b]
Thioridazine	515	116[c]

[a]Mean of 3–7 determinations.
[b]In-house determination.
[c]Guo and Guthrie *(10)*.
[d]Redfern et al. *(9)*.

4. Conclusions

Automated patch clamping using planar patch technology combined with sophisticated liquid handling offers the ability to greatly increase throughput with minimal sacrifice in data quality. We routinely complete concentration–response curves for 25–30 cells in a single day, roughly four times the throughput of conventional methods. The voltage-dependent block of certain classes of compounds dictates that full voltage control is important for characterising the physiological effects of drug application. This has been found to be very important for measuring drug block of hERG *(18)*. An example was shown for human Nav1.2 where compounds were found to cause a shift in the voltage dependence of channel inactivation. It is possible to apply the vast majority of voltage manipulations in an automated format using the QPatch. The results confirm the utility of automated patch clamping for investigating complex aspects of voltage-gated ion channel function and pharmacology.

5. Notes

1. ATP should be added to the internal solution from dry powder at the start of each day to avoid rundown of hERG current.

2. Based on experiments considering modulation of pH and osmolarity of the SFM II storage medium during the 4 hours on the QPatch, we recommend the use of 25 mM HEPES in the SFM II storage medium. We have found that addition of 25 mM HEPES modulated the pH value to a more physiological value (7.4), and it increased the osmolarity of the SFM II storage medium to a more physiological level (297 mOsm). Finally, application of 25 mM HEPES did not reduce the gigaseal formation rate or the whole-cell establishment.
3. For some series of compounds it is important to use glass-coated vessels to avoid adsorption to plastics.
4. Drug additions to the chip units are made at least twice in order to ensure that complete equilibration occurs to the required drug concentration.
5. If confluency is anticipated to be higher than 80% on the day of the experiment, flasks can be placed in an incubator at 30°C for 24 hours. This slows growth and can improve surface expression for some cell lines.

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