RESEARCH NOTE

A nonlinear and time-dependent leak current in the presence of calcium fluoride patch-clamp seal enhancer [version 1; peer review: 4 approved with reservations]

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Abstract

Automated patch-clamp platforms are widely used and vital tools in both academia and industry to enable high-throughput studies such as drug screening. A leak current to ground occurs whenever the seal between a pipette and cell (or internal solution and cell in high-throughput machines) is not perfectly insulated from the bath (extracellular) solution. Over 1 GΩ seal resistance between pipette and bath solutions is commonly used as a quality standard for manual patch work. With automated platforms it can be difficult to obtain such a high seal resistance between the intra- and extra-cellular solutions. One suggested method to alleviate this problem is using an F^- containing internal solution together with a Ca^{2+} containing external solution — so that a CaF_2 crystal forms when the two solutions meet which 'plugs the holes' to enhance the seal resistance. However, we observed an unexpected nonlinear-in-voltage and time-dependent current using these solutions on an automated patch-clamp platform. We performed manual patch-clamp experiments with the automated patch-clamp solutions, but no biological cell, and observed the same nonlinear time-dependent leak current. The current could be completely removed by washing out F^- ions to leave a conventional leak current that was linear and not time-dependent. We therefore conclude fluoride ions interacting with the CaF_2 crystal are the origin of the nonlinear time-dependent leak current. The consequences of such a nonlinear and time-dependent leak current

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polluting measurements should be considered carefully if it cannot be isolated and subtracted.

Keywords
electrophysiology, leak current, automated patch, patch clamp, seal enhancer

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Introduction

Voltage-clamp and current-clamp have been vital tools for studying electrophysiology since the time of Hodgkin & Huxley (1952). Voltage-clamp experiments are commonly used to study voltage and time dependence of ion currents; current-clamp experiments are used to study for example action potentials of excitable cells. Many different techniques have been developed and one of most the widely-used methods is whole-cell patch clamping (Sakmann & Neher, 1984).

Whole-cell patch-clamp experiments can be performed using either manual control of a pipette's position or on automated high-throughput machines based on microfluidics. Manual patch is the conventional method, but it can be very time consuming and low-throughput; whilst automated platforms allow high-throughput recordings, which can be extremely useful for studies that require high numbers of measurements such as drug screening in the pharmaceutical industry (Elkins *et al.*, 2013). In recent years many studies have begun to use automated patch-clamp systems to study ion channel electrophysiology (Gertler *et al.*, 2019; Kang *et al.*, 2019; Kozek *et al.*, 2020; Lei *et al.*, 2017; Lei *et al.*, 2019a; Lei *et al.*, 2019b; Li *et al.*, 2017; Ng *et al.*, 2020; Toh *et al.*, 2020; Vanoye *et al.*, 2018).

A schematic comparison of the two patch-clamp methods is shown in Figure 1A–B. Manual patch-clamp uses a fire-polished glass pipette to form a tight electrical seal (~GΩ) between the pipette tip and the cell membrane. Although the composition of the ionic solutions in the pipette and bath depends on the type of experiments, these solutions are usually intended to be similar to the relevant physiological conditions. Automated platforms, on the other hand, usually have a very different configuration to manual patch, see Figure 1B; they use a design where the cells are suspended on top of a micro-pore on a planar surface. However, this planar design does not always yield as tight a seal (~ hundreds of MΩ) as the conventional manual patch-clamp. Seals can be enhanced in the presence of certain additional ions in the two solutions. For instance, a F^- containing internal solution together with a Ca^{2+} containing external solution has been used with manual patching (Kostyuk *et al.*, 1975; Tasaki & Takenaka, 1964), and is used with many automated platforms (Kramer *et al.*, 2020). The improvement of seal resistance with these solutions is thought to be due to the formation of CaF_2 crystals at the interface between the pipette or micro-pore and the cell.

Many studies have compared manual patch clamping with automated patch clamping data, and showed that their performances are similar (Billet *et al.*, 2017; Lei *et al.*, 2019a; Lei *et al.*, 2019b; Li *et al.*, 2017). Here we examine a difference in the kinetics (dynamics) of the leak currents that can be observed between the two platforms.

Figure 2A shows an automated patch (Nanion SyncroPatch 384PE) recording of the leftover current measured on Chinese hamster ovary (CHO) cells transfected with the human Ether-à-go-go-Related Gene (hERG)1a after applying a hERG-specific blocker (in this case 0.5 μM of E-4031) at 25 °C. One might assume this remaining current consists of both leak current (due to finite resistance of the seal) and/or ion currents conducted by non-hERG ion channels natively present in the CHO cells (which we refer to as 'endogenous currents'). The measurements (blue) are consistent across laboratories. In Figure 2 we show measurements taken at: (A) F. Hoffmann-La Roche, Basel (Lei *et al.*, 2019a; Lei *et al.*, 2019b); and (B) Victor Chang Cardiac Research Institute in Sydney (using the same type of Nanion SyncroPatch machine).

The leftover currents are time-dependent when the cell is clamped at a constant voltage; their current-voltage (I-V) relationships (at steady state) are non-Ohmic (nonlinear), as shown in Figure 2 (right panels) blue crosses. Therefore we refer them to as 'nonlinear time-dependent' currents. At first

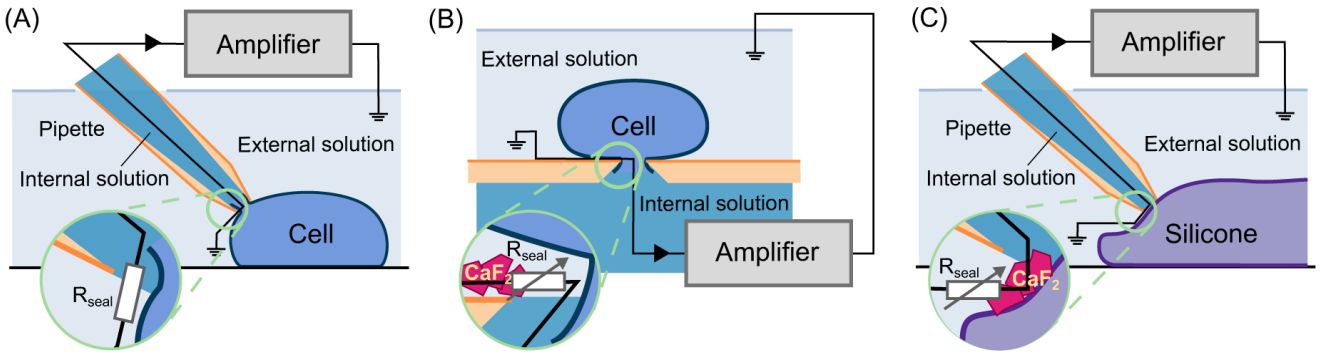

Figure 1. A schematic comparison of manual and automated patch-clamp methods, with a cartoon representation of the leak current circuit. (**A**) Shows the conventional manual patch-clamp, where a polished glass pipette is used to form a tight electrical seal. (**B**) Shows the planar design of an automated patch-clamp, where the cell is suspended on top of a micro-pore in the presence of CaF_2. (**C**) Shows the set-up of our manual patch-clamp silicone experiments with automated patch-clamp solutions. The magnifications show the difference between the leak current from the three configurations.

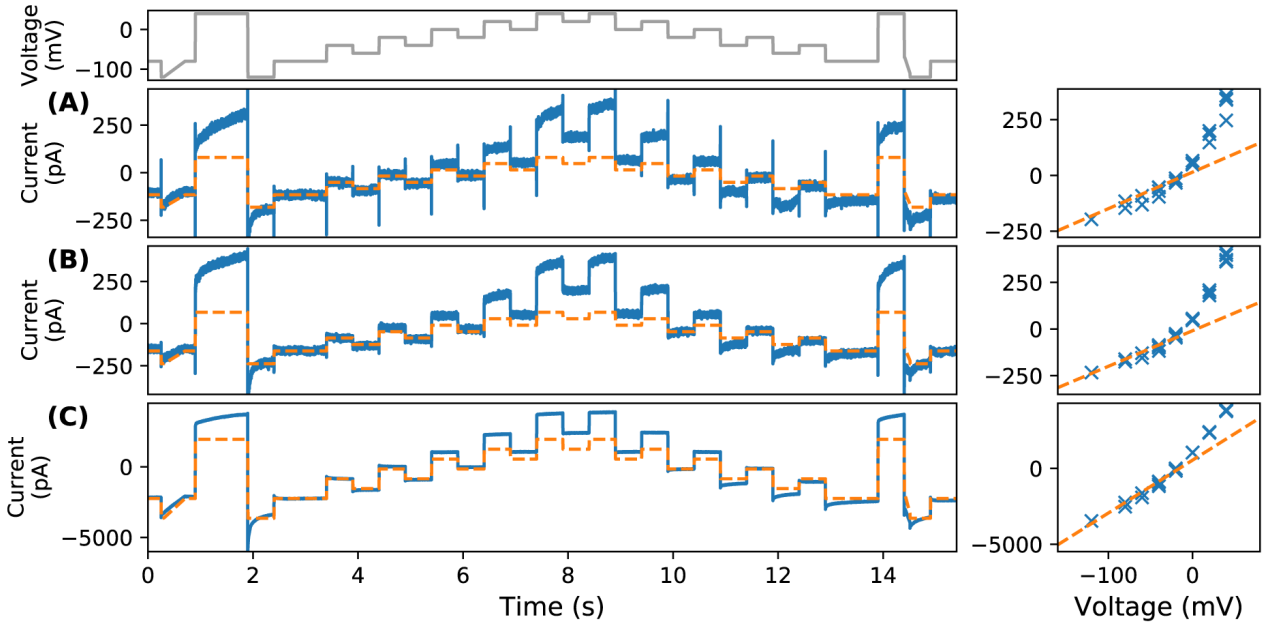

Figure 2. Examples of nonlinear time-dependent leftover current in an automated patch-clamp platform. On the left shows the time series recordings under the staircase protocol (top panel) (Lei *et al.*, 2019a); right shows the current-voltage (I-V) relationships (at steady state). Experimental recordings are shown in blue, and linear leak estimations are shown in dashed orange. Typical recordings of the leftover current measured on hERG1a transfected CHO cells after applying an *I*Kr-specific blocker (0.5 μM of E-4031) are shown, where experiments were performed at (**A**) F. Hoffmann-La Roche, Basel (Lei *et al.*, 2019a; Lei *et al.*, 2019b) and (**B**) an independent reproduction at Victor Chang Cardiac Research Institute in Sydney. (**C**) Shows a typical recording for an empty well-plate (no biological cells in the solution) in the automated platform.

glance, they were thought to be endogenous biological currents given that leak current usually has a linear Ohmic form:

$$I_{\text{leak}} = g_{\text{leak}} \cdot (V_{\text{m}} - E_{\text{leak}}), \quad (1)$$

where V_{m} is the membrane voltage, and g_{leak} and E_{leak} are the maximum conductance and reversal potential of the leak current. We illustrate the shape of a linear leak current given by Equation (1) by overlaying the fitted current (see Methods section) as an orange dashed line in Figure 2. Note that the leak current here is assumed to be through an imperfect seal, instead of current through some 'leak channels' in the cell. Unlike the commonly-used human embryonic kidney (HEK) cells, CHO cells are thought to have little endogenous current (Yu & Kerchner, 1998).

The first question was whether the nonlinear time-dependent leftover current was endogenous current through native ion channels expressed in CHO cells. Figure 2C shows an empty well-plate experiment performed on the automated patch-clamp system (recorded as part of the study by Lei *et al.*, 2019a). That is, the experiments in Figure 2A–B were repeated without adding in any biological cells. The recording shows a similar current except with a much larger amplitude (due to an open chip there is very low seal resistance and an enormous leak current). This observation suggested the leftover current might not be endogenous current. The question then, is what causes the nonlinear time-dependent leftover current observed in automated patch-clamp platforms?

Understanding the origin of the nonlinear time-dependent leftover current is crucial for accurate use of recordings. If one uses a linear, non-time dependent leak correction (Equation (1)) the remaining nonlinear and time dependent current could 'look like' a real ion channel current, contaminate the recording, and lead to an incorrect characterisation of ion channel kinetics. Moreover, in the absence of any leak correction, the nonlinear leak current will also obscure the ion current of interest.

One way to reduce this effect is to use post-blocker subtraction. That is, after measuring the complete current, apply a specific and approximately complete block of the current of interest (e.g. blocking hERG with dofetilide or E-4031) and remeasure the leftover current; the difference between the two recordings should be mainly the current of interest. We used this subtraction method in previous studies where we first observed this non-linear leak (Lei *et al.*, 2019a; Lei *et al.*, 2019b). Even then, with post-blocker subtraction the seal resistance can change over time (especially when a relatively long time period is needed for a blocker to have full effect, or

long protocols are required) and the subtraction method will not be able to fully remove the nonlinear time-dependent leak current. Studies without a specific-blocker subtraction method will suffer from polluted currents.

In this study, we examine the origin of the observed nonlinear time-dependent leftover current in automated patch-clamp platforms.

Methods

The observation in Figure 2C suggested the leftover current might not be endogenous current, and motivated further investigations using the F^- containing and Ca^{2+} containing automated patch-clamp solutions in a manual patch experiment.

Voltage-clamp experiments were performed in the same way as the conventional manual patch except the cell was replaced with a silicone elastomer ('SYLGARD'), as shown in Figure 1C. Using this approach we ensured that: (1) the behaviour of the measured current was not caused by the planar-micro pore configuration (Figure 1B) or anything specific to the automated platform; and (2) any observations must *not* be uncontrollable endogenous biological currents, but purely an electro-chemical phenomenon, as there were no biological cells involved.

A conventional manual patch-clamp system (HEKA EPC 10 USB Single, HEKA Elektronik GmbH, Lambrecht/Pfalz, Germany) was used for the voltage-clamp experiments. As the pipette tip was gradually moved closer to the silicone elastomer, a seal resistance in the range of 100–1000 MΩ could be obtained, similar to for example Lei *et al.* (2019a); Lei *et al.* (2019b), such that a magnitude of leak current could be measured that was similar to the biological measurements. Leak current between the pipette and the silicone with various patch-clamp solutions was measured and compared to the currents shown in Figure 2A–B.

All codes and data are freely available (see data and software availability Lei & Mirams (2020)).

Patch-clamp solutions

Three patch-clamp solutions were prepared: (1) Ca^{2+} containing automated patch-clamp external solution; (2) F^- containing automated patch-clamp internal solution; (3) no-F^- manual patch-clamp internal solution. The concentrations of the solutions are given in Table 1, all substances were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The automated patch-clamp solutions are the same as those used in Lei *et al.* (2019a); Lei *et al.* (2019b), which are very similar to other automated patch-clamp studies such as Kozek *et al.* (2020); Ng *et al.* (2020) and those suggested by Nanion for SyncroPatch platforms.

Silicone elastomer

The silicone elastomer (SYLGARD 184, The Dow Chemical Company) was prepared using a standard 10:1 ratio of base and catalyst. A thin layer of mixed elastomer was dispensed in 35 mm tissue culture dishes (product number 430165, Corning), and was cured at 60 °C.

Experimental procedure

Table 2 summarises the three sets of measurements that were performed. The currents were measured under a voltage-clamp protocol used in Lei *et al.* (2019a); Lei *et al.* (2019b), known as the "staircase protocol", shown in Figure 2; a time series file for the protocol is provided (see data availability Lei & Mirams (2020)). The holding potential was set to 0 mV.

Table 1. The patch-clamp solutions used in the experiments. All concentration are given in mM and product numbers refer to Sigma-Aldrich catalogue. Ca^{2+} containing (automated patch external) solution was titrated to pH 7.4 with HCl; F^- containing (automated patch internal) and no-F^- (manual patch internal) solutions were titrated to pH 7.2 with KOH.

Solutions Product number	NaCl S9625	KCl P4504	KF 449148	$MgCl_2$ 63069	$CaCl_2$ 21113	HEPES 54457	Glucose G8270	NMDG 66930	Sorbitol S1876	MgATP A9187	EGTA E4387
(1) Ca^{2+} containing	97.5	4	—	1	**2.05**	10	5	35	20	—	—
(2) F^- containing	10	10	**100**	—	—	10	—	—	—	—	20
(3) No-F^-	—	130	—	1	—	10	—	—	—	5	5

Table 2. Summary of the three sets of voltage-clamp measurements performed using a manual patch-clamp system with silicone elastomers. Measurement III was performed by washing out the externally applied F^- containing solution in Measurement II with the no-F^- solution, the aim was that the measurements were done in the presence of the CaF_2 crystal but without F^- in solution.

Measurement	Internally applied solution	Externally applied solution
I	(2) F^- containing	(1) Ca^{2+} containing
II	(1) Ca^{2+} containing	(2) F^- containing
III	(1) Ca^{2+} containing	(3) No-F^-

Measurement I is the 'standard' configuration that one would use in an automated platform (the same as in Figure 2), which aimed to reproduce the nonlinear time-dependent leftover current observed in automated platforms. Measurement II investigates the current's dependence on the ionic solutions by swapping the internal and external solutions. Measurement III is the control experiment by washing out the F^- containing solution in Measurement II with the no-F^- solution; it was performed after Measurement II, where the F^- containing solution was the external solution and could be easily changed.

Data analysis

We estimated g_{leak} and E_{leak} in Equation (1) using two voltage steps (−80 mV and -40 mV unless otherwise specified); g_{leak} was estimated by the ratio of the voltage difference and the mean current difference using the last 500 ms of the voltage steps, after which E_{leak} can be directly calculated from Equation (1) using one of the voltage steps. The steady state of the nonlinear time-dependent leak current at each voltage step was estimated by fitting a single exponential of the form: $a\times\exp(-b(t - t_0))+c$, where a, b, c are the parameters to be fitted and t_0 is the starting time of the voltage step. The first 5 ms at the beginning of each voltage step was ignored due to the capacitance spike. The parameter c is the estimated steady state of the leak current of the given voltage step. All the analysis was performed in Python using NumPy/SciPy (Jones *et al.*, 2001); all the code for the analysis is provided (see software availability Lei & Mirams (2020)).

Results

Measurement I

The recorded leak current for Measurement I is shown in Figure 3I. The leak current measured with silicone elastomer replicates the leftover current observed using automated patch-clamp platforms after blocker-application in CHO cells. Not only were the size of the currents comparable (a few hundred pA), but also the pattern and dynamics were extremely similar to those seen in automated platforms at multiple sites (Figure 2A–B). The measured leak current was time-dependent when it was held at a constant voltage, and it showed a noticeable outward (positive) time-dependent current during an increase of voltage from zero to 40 mV. Furthermore, the I-V relationship (at plateau) was non-Ohmic (nonlinear).

Measurement II

In this set of measurements, we repeated the experiments but swapped the internal and external solutions in Measurement I. Figure 3II shows the recorded leak current for Measurement II. Both the time-dependent part of the leak current and its nonlinear I-V relationship were reversed. Instead of a prominent outward (positive) time-dependent current during an increase of voltage from approximately 0 to +40 mV, a noticeable inward (negative) time-dependent current was produced during a decrease of voltage to approximately −80 to −120 mV. Moreover, the nonlinear I-V relationship (at plateau) changed from superlinear in Measurement I to sublinear, as shown in the right panels of Figure 3I–II. Note that, as the nonlinearity was thought to be caused by the inward time-dependent current

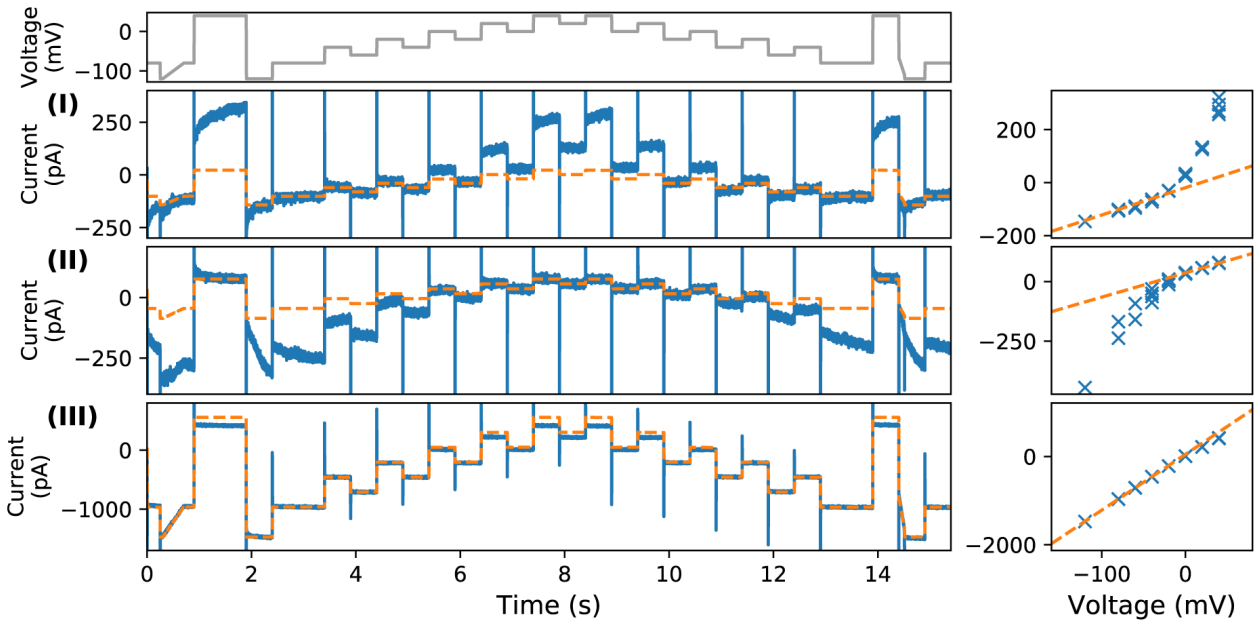

Figure 3. Conventional manual patch-clamp recordings with automated patch-clamp solutions on silicone elastomer. On the left shows the time series recordings under the staircase protocol (top panel) from Lei *et al.* (2019a); right shows the current-voltage relationships (fitted with a single exponential function, approximating the steady state relation). Experimental recordings are shown in blue, and linear leak estimations are shown in dashed orange. (**I**)–(**III**) show the results of Measurements I–III in Table 2. (**I**) with internal and external solutions as per automated experiments (similar to those in Figure 2); (**II**) with internal and external solutions swapped, the nonlinear portions are now at negative rather than positive voltages; (**III**) same conditions as (**II**) after F^- was washed out of the bath — the current becomes much closer to the expected linear leak given by Equation (1).

at low voltage, the linear leak estimation (orange dashed line) in Figure 3II was fitted to two voltage steps at higher voltages (+40 mV and +60 mV).

Measurement III

Finally, immediately following Measurement II during the same experiment the externally applied F^- containing solution was washed out and replaced with the no-F^- solution externally. Note that the measurement was performed by washing the external solution in Measurement II after the crystal was formed, and therefore the measurement should be in the presence of the CaF_2 crystal, although the magnitude of the current became larger due to the wash. The results are shown in Figure 3III. The nonlinear I-V relationship and time-dependent dynamics of the leak current were almost entirely eliminated; linear leak current that follows Equation (1) was observed by simply removing the F^- containing solution.

Discussion

In this study, we observed a nonlinear and time-dependent current in an automated patch-clamp platform, in this case the Nanion SyncroPatch, whilst taking recordings from CHO hERG1a cells in the presence of a hERG blocker. We investigated the origin of this 'leftover current' as it is crucial for accurately determining the kinetics of ion channel currents (Lei *et al.*, 2020). Experiments using a conventional manual patch-clamp setup on a silicone elastomer were performed with automated patch-clamp solutions.

Our results (Measurement I) show that it was possible to replicate the nonlinear time-dependent leftover current in automated platforms (Figure 2A–B) with manual 'no-cell' (silicone elastomer) experiments (Figure 3I). Therefore, the leftover current cannot be an endogenous current from the (CHO) cells, and is predominantly a leak current through the imperfect seal. We then show that by interchanging the internal and external solutions (Measurement II), the time-dependence was retained but the nonlinear I-V relationship of the current was reversed (Figure 3I–II). This is evidence that the nonlinear time-dependent part of the leak current is determined by the two ionic solutions used. Finally, in Measurement III (Figure 3III), the nonlinear I-V relationship and time-dependent dynamics of the leak current were eliminated by washing the externally applied F^- containing solution in Measurement II and replacing with the no-F^- solution externally. Note that the measurement was performed by washing the F^- containing solution in Measurement II after the crystal was formed, hence the nonlinear time-dependent leak current occurred as a consequence of the presence of the crystal *and* fluoride. This clearly demonstrates that the cause of the I-V nonlinearity and time-dependent behaviour of the leak current is the F^- containing internal solution used as part of a seal enhancer in automated patch-clamp systems.

We propose the following tentative hypothesis to explain the observed I-V nonlinearity and time-dependent behaviour of the leak current. Our leading conjecture is that the nonlinear time-dependent leak current consists of two parts, one is the ordinary linear leak through imperfect seal and the other one is an 'extra' current that behaves nonlinearly and time-dependently. The basis of this 'extra' current could be defects in the CaF_2 crystals. The anion vacancies created in the crystal lattice lead to net positive charge (Huisinga, 1999), which acts as a charged plug (similar to e.g. polyamines blocking inward rectifier channels) giving the observed asymmetric leak. Furthermore, F^- has a higher mobility than Ca^{2+}, so F^- may preferentially move out through the imperfect seal and form crystals with Ca^{2+} on the Ca^{2+}-side of the membrane. This hypothesis is consistent with the direction of the 'extra' current in the leak in both Measurements I and II.

Our findings have implications in methods for measuring and post-processing the recordings. The leak current has a nonlinear I-V relationship and time-dependence when it is held at a constant voltage, it is therefore important to subtract it off from the recordings such that a pure current of interest can be obtained. Due to its nonlinearity and time-dependence, the kinetics of the resulting current of interest (hERG1a current in our examples) could be undesirably affected if not carefully removed: the fluoride-dependent leak current can shift the I-V curve of measured currents, alter observed time constants, etc.

Since the form of this leak current is, to our knowledge, not very well studied; it is not possible to use the standard methods of estimating linear leak current to perform the correction. The standard methods involve a small leak step (change in voltage) at which the ion channel of interest is (nearly) closed. However, given the nonlinearity in the I-V relationship of this current, a voltage-current estimation across one range or pair of voltages would result in an incorrect estimation of the whole I-V relationship (see for example how the orange dashed lines missed the blue crosses in Figure 2 and Figure 3 right panels), and the time-dependent dynamics would not be captured. The best approach available at present is the widely-used block-and-subtract method, as used in our earlier studies Lei *et al.* (2019a); Lei *et al.* (2019b). However, the seal can change over time (especially if a relatively long period is allowed for a blocker to take effect). In which case, the subtraction will not be able to completely remove the nonlinear time-dependent portion of the leak current, resulting in over- or under-subtracted leak current. Therefore this study raises concerns about the effects and consequences of this nonlinear time-dependent leak current.

There are two obvious options to account for this current. Firstly, and ideally, we would completely remove this nonlinear time-dependent leak current by altering the ionic solutions. We have seen that the properties of the current depend on the concentration of F^- in the solutions (and will probably also depend on $[Ca^{2+}]$). Optimal concentrations for these ions may exist that are high enough to sufficiently enhance the seal, but low enough that the nonlinearity and time-dependence are not evident in recordings. Other salts such as BaF_2, $CaSO_4$, etc. should be tested to see whether they can enhance seals but remove this nonlinear time-dependent leak current. It may also be possible to wash away the F^- ions after establishing a seal.

Secondly, further studies of this F^--dependent current could allow it to be modelled so well that it could be subtracted from recordings in post-processing in much the same way as the linear leak. But at a minimum this option would involve: a better characterisation of the time and voltage dependence of this leak current; its dependence on the concentration of F^- and/or Ca^{2+} in the solutions; dependence on the seal resistance; and testing that the current is predictable (and not, for example, a function of unmeasured quantities such as crystal size/thickness/volume). We anticipate that removing the current through alterations to the ionic solutions will be simpler and more reliable.

Conclusions

We observed a nonlinear and time-dependent current in automated patch-clamp platforms in the presence of Ca^{2+} and F^- (intended to form a CaF_2 seal enhancer). The same nonlinear time-dependent current was observed using a conventional manual patch-clamp setup in close proximity to a silicone elastomer to form a seal between 100–1000 MΩ. Therefore the current was determined to be mainly leak current through imperfect seal, and not endogenous current from the cells. The nonlinear and time-dependent form of the leak current was caused by the presence of CaF_2 and could be eliminated by F^- washout after CaF_2 crystal formation.

Data availability

Underlying data

All datasets used in the publication are available at: https://github.com/CardiacModelling/nonlinear-time-dependent-leak.

A permanently archived version is available on Zenodo: https://doi.org/10.5281/zenodo.3876262 Lei & Mirams (2020)

This project contains the following underlying data:

- `data/protocol-staircaseramp.csv` — a time series trace of the voltage protocol.
- `data/silicone` — a set of voltage-clamp timeseries data in HEKA format for Measurements I, II and III; as plotted in Figure 3)
- `data/cho-cell` — a set of CHO-hERG cell voltage-clamp time-series data taken from (Lei *et al.*, 2019a), as plotted in Figure 2.
- `data/no-cell` — a set of empty well-plate voltage-clamp time-series data taken from (Lei *et al.*, 2019a), as plotted in Figure 2.

A description of other files, including python scripts to read and plot these data, is available in the repository Readme file.

Software availability

Source code is also available from: https://github.com/CardiacModelling/nonlinear-time-dependent-leak and was archived at time of publication: https://doi.org/10.5281/zenodo.3876262 Lei & Mirams (2020)

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Current Peer Review Status: ? ? ? ?

Version 1

Reviewer Report 30 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39340

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Carlos Guillermo Vanoye
Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

In the present manuscript, Lei *et al.* describe a rectifying, time-dependent leak current that is fluoride-dependent. This is well written manuscript with very interesting results. Their observation is important because some high throughput systems require the presence of fluoride to enhance seal formation. And the presence of this current, if not corrected properly, could contaminate ion channel recordings complicating results interpretation.
Comments:

1. Were the current traces shown in Figs 2 and 3 recorded with capacitance and series resistance compensation turned on? The compensations may affect the shape of the observed current.

2. The seal resistance values for the recordings shown in Figs. 2 and 3 are not given. Those values would allow the reader to interpret the amplitude of the leak current shown. This information is critical specially when comparing the results in Figure 3.II and Figure 3.III.

3. Each of the I-Vs shown in Figures 2 and 3 appear to be derived from only one recording. Please provide data from multiple wells (Fig 2) and patches (Fig 3) and show means, standard errors (or standard deviations) and statistical significance. The conclusions would be supported by providing I-Vs derived from multiple observations not just from one.

4. The slope of the outward current in Fig.2A and 2B is steeper than the one shown in Fig 2C. It is assumed that no currents are going through the cell membrane on those recordings but this may not be the case. Is the block of hERG1a by E-4031 100%? There could also be chloride channels (Gill *et al*, 2006)[1] that may be carry the observed current. The conclusion that there is no current going through ion channels in those recordings would be strengthened if cesium was used instead of potassium (or using non-transfected CHO-K1 cells) and chloride channel blockers were added.

5. Regarding the results shown in Figure 3, the authors should compare their observations to

those previously reported by Sachs and Qin (1993)[2]. The previous results showed ion-selective currents in the absence of CaF_2 using a similar approach (glass pipette plus Sylgard).

6. Is the linearity of the IV curve shown in Fig. 3.III due to the absence of fluoride or to the large drop in seal resistance? Please provide pre- and post-wash seal resistance values. Also multiple recordings ("patches") would be recommended.

7. Is the drop in resistance due to the loss of fluoride (and presumably CaF_2 crystals)? Or to the pipette and Sylgard moving apart during the wash? Performing a wash with a fluoride-containing solution could answer this question.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Ion channels, electrophysiology, molecular biology, cell physiology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 15 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39338

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Marc Rogers
Metrion Biosciences Limited, Cambridge, UK

Lei *et al.* expand on a historical automated patch clamp (APC) study of hERG current kinetics with a replicate APC study from a collaborator laboratory, to confirm the evidence for a nonlinear time- and voltage-dependent current component of unknown origin that would be problematic to subtract or remove using traditional pharmacological or biophysical techniques. In an effort to determine the basis of this non-linear current they emulate it using manual patch pipette recordings from an artificial silicone 'cell', concluding that the combination of a high (internal) concentration of fluoride ions and a physiological concentration of (external) Ca2+ ions either side of a membrane seal produces a CaF crystal that can lead to non-linear current in APC recordings.

- Is the work clearly and accurately presented and does it cite the current literature?

Yes

- Is the study design appropriate and does the work have academic merit?

Partly – lack of cell-based data to back-up claims made using artificial silicone cell.

- Are sufficient details of methods and analysis provided to allow replication by others?

Yes

- If applicable, is the statistical analysis and its interpretation appropriate?

Partly – additional replicates, means and statistical variation data would be useful.

- Are all the source data underlying the results available to ensure full reproducibility?

Yes

- Are the conclusions drawn adequately supported by the results?

Partly - see below.

Starting in the Abstract, the authors fail to adequately discriminate and make clear that their findings, and technical data, do not apply to all automated patch clamp platforms. It is neither fair nor accurate to paint all APC platforms with the same brush, as seems common in some academic papers and comments on this technology. Each APC platform is different, and their experience and main point about the combination of internal fluoride and high external divalent 'seal enhancer' being obligate to achieving gigaohm seals primarily applies to a single APC platform manufacturer.

The second major issue is that the authors seem to suggest that a combination of high internal fluoride and external divalent cations is required to acquire gigaohn seals on APC platforms, and again this is inaccurate and may reflect their lack of experience with multiple platforms. It is true that some APC platforms may rely on high internal fluoride to achieve a high frequency of gigaohn seals, but this is neither obligate nor common these days, and it is actually contraindicated to

combine high internal fluoride and elevated external divalents for many APC platforms.

Also, the authors just tested a single combination of internal F and external Ca^{2+} (Table 1), but should and could have looked at several different combinations and concentrations (as suggested in the Discussion) to determine the true source and magnitude of the CaF effect. In addition, many groups can achieve gigaseals without using >100 mM internal fluoride through a combination of optimised cell culture and experimental conditions, and biocompatible chip substrates. Thus, the statements in the Abstract that "*With automated platforms it can be difficult to obtain such a high seal resistance between the intra- and extra-cellular solutions*" and "*One suggested method to alleviate this problem is using an F containing internal solution together with a Ca containing external solution — so that a CaF crystal forms when the two solutions meet which 'plugs the holes' to enhance the seal resistance*", and the schematic and legend to Fig. 1B, are inaccurate and incorrect.

As an APC user myself and a decades long patch clamper, I also have difficulty accepting the implication that the use of high internal fluoride and even low mM external divalents is a 'trick' solely employed for gigaseal APC recordings. I have lost track of the number of peer reviewed publications on voltage-gated Nav channels from leading academic groups, for example, that use this exact same recipe for their manual patch recordings, largely to ensure high resistance high fidelity recordings. Fig. 1A ignores this well-known tradition, assigning manual patch gigaseal resistances to the pipette glass-membrane tight seal alone. Thus, I would expect that a similar non-linear leak phenomenon would also be observable in many typical manual patch clamp recordings, but the authors notably did not run this experiment, and instead opted for a cell-free silicone-based biophysical manual patch pipette test. Thus, a claim or suggestion for 'correction' or close inspection of APC recordings employing a CaF effect would thus equally apply to a great number of past and future manual patch clamp datasets, but the authors do not include this possibility in their Abstract or Conclusions.

The authors assume that all time- and voltage-dependent, exogenously expressed hERG channel current is removed in the presence of the pharmacological blocker, but do not provide evidence that this is the case under their recording conditions. By inference they suggest this is the case, and thus the similarity between the remaining non-linear outward leak current (Fig. 2A, B) and the open chip APC recording (Fig. 2C) is due to the CaF2 effect, rather than remaining hERG conductance.

Similarly, the authors cite a reference on p4 that CHO cells have 'little endogenous current', but could have easily determined this empirically using wildtype or non-transfected cells and the same recording conditions and APC platforms used in the present study. Both sets of additional cell-based experiments would have bolstered their silicone 'cell' dataset, removing two possible related explanations for the APC cell non-linearity (leftover hERG and/or endogenous conductances, both of which would be expected to be time-dependent and non-linear at positive voltages), and strengthening their main claim about a non-linear CaF leak effect without relying on a simple 'they look the same' argument.

Obviously there is a CaF-mediated non-linear biophysical phenomenom seen in the silicone-cell manual patch experiments, but acceptance of this manuscript and their (modified/clarified) claims about this affecting certain APC platform recordings requires actual cell-based data to compliment their historical APC datasets.

Also, their Discussion suggestions at the bottom on p7 are somewhat out-of-date, as many of these options have already been explored by experienced APC users (e.g. reducing Fl and Ca concentrations, use of alternate cations).

Finally, the language in the Conclusions needs to be re-worded to limit their claims to a certain type of APC platform (i.e. remove the plural to 'APC platforms'). Also, the Conclusion stating that the CaF non-linear leak current is not due to endogenous (or non-blocked hERG) conductances is also not bourne out by the lack of manual patch clamp cell-based experimental data in this study, as outlined above.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Manual patch clamp, automated patch clamp, drug discovery screening, pharmacology, biophysics.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 14 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39339

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Andrew Glazer

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Lei *et al.* describe a non-linear leak current that appears in the presence of fluoride, an ion used in automated patch clamp instruments to enhance seals. This unexpected leak current, not modeled by traditional leak current linear adjustment calculations, could interfere with the accurate characterization of ion channel properties on these instruments. Using manual patch clamp experiments with a silicone model of a cell, they reproduce the “extra” leak current and demonstrate that it disappears when the fluoride is washed out.

The paper is clear and well-written, and describes an important finding that has implications for the growing field of automated patch clamp electrophysiology. The authors make the raw data and code available, and some of their observations were reproduced by 2 different laboratories/instruments.

Major comments: The authors propose one solution to this problem, which is to do a full blocker-subtraction for all measurements, which could indeed help remove this “extra” leak current. However as the authors note, the properties of the seal might change over the course of the blocker addition (and some protocols are even longer to carry out than their staircase protocol). But what about actually removing the current with an internal solution exchange - another option the authors briefly mention in the discussion? Could they generate the initial tight seal on the SyncroPatch using an fluoride-containing internal solution and calcium-containing external solution, then do an internal solution exchange that removes the fluoride? Would the seals remain strong and the extra leak current go away, as they saw with Measurement 3 in manual patch clamp? If successful, this could demonstrate a relatively easy solution to this problem on the SyncroPatch. The authors briefly propose this experiment in the discussion as a future direction but it would be a nice addition to this paper to demonstrate it.

Please present multiple replicate cells for the I-V curves and show means and standard errors.

Minor comments:
The staircase protocol is a nice alternative to the standard hERG protocols and is described in the methods. But it would be helpful to mention it briefly in the Introduction and give a citation to the papers by the same authors that developed it to avoid confusing readers who might be expecting standard hERG protocols.

The paper is best read linearly including fully reading the Methods to understand the logic of the silicone and the experimental measurements. However I worry readers might skip the methods and jump to the results where there is not much explanation/motivation for the 3 measurements. This could be solved by adding more motivating text to the start of the results and to the start of each experimental measurement paragraph. For example, the first two paragraph of the methods could be moved to become the first two paragraphs of the results. And a sentence or two could be added to the start of each measurement paragraph in the results to describe the aim/goal of the measurement, for readers who didn’t read or skimmed the Methods.

Comparing 3-II to 3-III, although the leak current now appears to be “normal” (extra leak current removed), is the magnitude of the leak current higher after the fluoride solution is washed out? Could the authors present data on the resistances/leak current magnitudes before and after

fluoride washout? If there is a less tight seal, would this prevent the success of the internal solution exchange strategy on the SyncroPatch because the seals would decrease following washout?

After some confusion, I realized that X's in the the I-V curves in figures 2+3 each show 1 cell from the staircase protocol, with multiple X's at the same voltage. Please clarify this in the legend.

The author's charged plug model for the leak current is a bit speculative - but it is caveated as a "tentative" model/hypothesis.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Automated patch clamping.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 13 July 2020

https://doi.org/10.21956/wellcomeopenres.17512.r39341

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Gildas Loussouarn
CNRS, INSERM,the thorax institute, University of Nantes, Nantes, France

In the present manuscript, Lei and collaborators report the observation of a rectifying and time-dependent current, after the generation of Giga-seals enhanced by CaF crystals, a procedure that is unavoidable for several automated patch-clamp platforms using the planar patch configuration. This observation is of interest since such current, if not removed, may pollute the current under study.

The authors replaced a living cell by a drop of Sylgard in a conventional patch-clamp set-up to mimic an imperfect seal. Using this original procedure, they could observe a CaF-induced leak current, in absence of any plasma membrane endogenous current. However, some complementary experiments should be added to give more insights on the CaF-induced current and avoid any over-interpretation of the data.

1. The panels (A) and (B) of Figure 2 show strikingly similar recordings from two different laboratories using the same solutions in the same type of Syncropatch machine. Average of currents, at least for the I/V curve would be more convincing than comparing two representative cells.
2. Panel (C) is quite different to panel (A) and (B), with similar time dependent currents generated by a large depolarization (-80 to +40 mV) but not by smaller depolarization (0 to +40 mV). In Syncropatch systems, to avoid over–compensation and current oscillations that can disrupt the seal, Rseries feedback compensation of the recorded current is applied with a slow time constant. Given the high current amplitude (several nA), is it possible that the time-dependent current observed in panel C is due to slowly developing feedback compensation?

3. Figure 3, when switching internal and external solutions, it would be more relevant to also invert the polarity of the voltage protocol and to directly compare the superimposed currents. Moreover, as in point 1, average of currents, at least for the I/V curve would be more convincing.

4. Experiments in Figure 2 and 3 are quite different, with, in Figure 2, an automated patch-clamp system and a real cell whereas in Figure 3, a conventional patch-clamp system and an artificial cell. There may some other models without endogenous current, that can be used in automated patch-clamp, such as giant unilamellar vesicles[1]. Another option would be to test if the currents observed in Figure 2 and 3 are of the same nature, by replacing internal K^+ and/or Na^+ by the organic cation N-methyl-D-glucamine (NMDG) in the intracellular medium (be very careful if you need to use HF to prepare this solution, HF is a very corrosive and extremely toxic acid) or by replacing Cl^- by gluconate in the extracellular medium. Indeed, since the time-dependent current is an outward current, it may be carried by cations diffusing from the intracellular medium to the extracellular medium and/or by anions diffusing from the extracellular medium to the intracellular medium. Fluoride diffusion is unlikely the basis of the observed current, as suggested in the discussion: "*Furthermore, F^- has a higher mobility than Ca^{2+}, so F^- may preferentially move out through the imperfect seal and form crystals with Ca^{2+} on the Ca^{2+}-side of the membrane. This hypothesis is consistent with the direction of the 'extra' current in the leak in both Measurements I and II.*"

Minor points

1. In 'Data Analysis', "*E_{leak} is directly calculated from equation (1) using one of the voltage step*": it would be interesting to indicate if E_{leak} calculations gives values close to zero.

2. In the results, sentence "*The measured leak current was time-dependent when it was held at a*

constant voltage, and it showed a noticeable outward (positive) time-dependent current during an increase of voltage from zero to 40 mV". I don't understand the difference between the two described time-dependence. I think the sentence could be simpler.

3. In the discussion: it is proposed that when fluoride is removed, CaF crystals remain, suggesting that both CaF and F^- need to be present to observe the time dependent and rectifying current. The fact that seals quality deteriorates upon F^- removal suggest that CaF crystals disappear, so the loss of the outwardly rectifying current may be due to the loss of crystals, and for instance, the loss of a rectifying current carried by intracellular cations in the crystal lattice, as suggested in "major point 4". There is no strong argument for the direct presence of fluoride as a cause of the outwardly rectifying current.

4. Authors should indicate other issues regarding the use of intracellular fluoride and extracellular calcium as a seal enhancer. Fluoride is (i) a phosphatase inhibitor and, obviously here, (ii) a calcium chelator preventing the use of calcium containing intracellular solutions. Using extracellular calcium at higher concentration than the physiological range alters channel gating[2], at least partly due to membrane charge screening[3].

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Electrophysiology, patch-clamp.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.