Characterisation of Schistosoma mansoni Glutamate-Gated Chloride Channels

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Running heading: **Characterising Schistosoma GluCl function**

Abstract

Electrophysiology is the standard method for characterizing ion channel function. Two electrode voltage clamp is a robust and relatively simple version which can be applied to the characterization of glutamate-gated chloride channels from *Schistoma mansoni*, a potential schistomicidal target. Here, the method is described in detail, with an emphasis on the investigation of *S. mansoni*. GluCls.

Keywords

GluCl, Schistosoma mansoni, ion channels, glutamate, receptor, pharmacology

1. Introduction

At the synapse and at the neuromuscular junction, neurotransmitter binds to and activates ligand-gated ion channels in the postsynaptic cell membrane. Na^+ and Ca^{2+} ion current through cation-selective channels leads to depolarization and thus excitation of postsynaptic cells, whereas Cl^- current through anion-selective channels generally hyperpolarizes cells or shunts depolarization and is inhibitory [1]. This conversion of neurotransmitter binding to excitation or inhibition is vital to animal function, and ligand-gated ion channels are common targets for pharmacological modulation. As glutamate-gated chloride channels (GluCls) occur in flatworms, such as *Schistosoma mansoni*, and are absent from vertebrates, they constitute a target for potential schistomicidal drugs [2].

The gold standard for functional and pharmacological characterization of specific ion channels is patch clamp electrophysiology, where channels are recombinantly expressed in cultured cells, and membrane currents in response to agonist and/or drug application are recorded [3]. A slightly easier method is two electrode voltage clamp, where cultured cells are replaced with larger, easier-to-handle oocytes from *Xenopus laevis* (African clawed frog) [4]. With the identification of GluCls and other ion channels from *S. mansoni*, schistomicidal drug discovery could benefit from electrophysiological and pharmacological characterization of these channels. This chapter serves to describe the characterization of GluCls by two electrode voltage clamp.

2. Materials

Electrophysiology set-ups can be pieced together with components from various suppliers but are often built around the main components of amplifier and digitizer from a particular supplier. The following outlines the set-up commonly used by this author, much of which is part of a convenient two electrode voltage clamp package, TEV-700 (Warner Instruments), but equivalent components are also available from e.g. Molecular Devices and NPI Electronic.

2.1 Two electrode voltage clamp set-up

1. Amplifier (OC-725C, Warner Instruments, includes bathclamp headstage)
2. Digitizer (Axon Digidata 1550B, Molecular Devices)
3. Acquisition software (pClamp 11, Molecular Devices)
4. Computer (see Note 1)
5. Baseplate (BPM-1, Warner Instruments, but see Note 2)
6. Stereomicroscope and stand (Z850, Unitron, see Note 3)
7. Oocyte recording chamber (RC-3Z, Warner Instruments)
8. Perfusion system (VCS-8-Pinch, Warner Instruments)
9. Waste collection system (DWV, Warner Instruments)
10. Left and right micromanipulators (MM-33L and MM-33R, and MB/B magnetics stands, Warner Instruments)
11. Electrode holders (E45W-F15VH for current cable and ESW-F15V for voltage probe, Warner Instruments)
12. Silver wire
13. Manoeuvrable light source

2.2 Other equipment

1. Micropipette puller (P-1000, Sutter Instruments)

2. Glass capillaries (Item 504949 from World Precision Instruments for RNA injection, GC120T-10 from Harvard Apparatus for two electrode voltage clamp)

3. Tweezers (Dumont Teezers #5, 0.1 x 0.06 mm tips, World Precision Instruments)

4. Microinjector (Nanoliter 2010, World Precision Instruments, this also requires a stereomicroscope and micromanipulators, much like the two electrode voltage clamp set-up)

5. MicroFil needles (MF28G-5, World Precision Instruments)

6. Parafilm

7. Disposable lint-free cloth wipes (Kimtech, Kimberly-Clark)

8. Grooved plate or wire grid. This is a simple plastid disc with grooves or a wire mesh/grid for holding oocytes conveniently when microinjecting (Fig. 1a). These can often be built in-house.

9. *X. laevis* stage V-VI oocytes (Ecocyte Bioscience, but see Note 4)

10. Data analysis software (Prism 8, GraphPad)

2.3 Reagents

1. Mineral oil

2. T7 or SP6 capped RNA transcription kits (Ambion mMMESSAGE mMACHINE, ThermoFisher Scientific)

3. RNeasy Mini Kit (Qiagen)

2.4 Solutions

1. OR2: 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4 with NaOH.

2. Leibovitz's L-15 medium (see https://www.thermofisher.com/no/en/home/technical-resources/media-formulation.80.html), supplemented with 3 mM L-glutamine, 2.5 mg/ml gentamycin, 15 mM HEPES, pH 7.4 with NaOH.

3. ND96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4 with NaOH.

4. ND96/gluconate: 96 mM Na gluconate, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4 with NaOH.

5. ND96/choline: 96 mM choline Cl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4 with NaOH.

3. Methods

3.1 Injection of RNA into oocytes

1. GluCl cDNAs should be cloned into a plasmid vector downstream of T7 or SP6 sequence (for recognition by polymerase in mRNA transcription), *X. laevis* β-globin promoter sequence, and Kozak sequence, and upstream of a poly(A) tail and then a restriction site for DNA linearization. Capped mRNAs are transcribed using kits. This author then purifies mRNA with columns in a kit, avoiding chemicals such as phenol/chloroform and LiCl. By eluting with a small volume, one typically recovers 10-20 µl RNA at > 1 µg/µl from a single reaction, which can be stored at -20 °C for days or at -80 °C for months or even years.

2. Protocols for pulling micropipettes vary with different filaments in the micropipette puller, and the P-1000 manual or Sutter micropipette cookbook describes how to establish these protocols. For injection of mRNA, a ~10 mm, string-like taper is desirable, the tip can be broken off with tweezers or fine scissors, achieving a tip small enough to hold RNA, but large enough to expel mRNA when required.

3. The microinjector should be set to the appropriate volume (see Note 5). Micropipettes are back-filled with mineral oil using a MicroFil needle and syringe, and the micropipette is inserted to the injector. A lint-free cloth wipe should be kept on hand for removing expelled mineral oil, as excessive oil on gloves, etc becomes troublesome. A one to two µl drop of mRNA (see Note 5) is pipetted on to a piece of Parafilm or an upside-down lid from a 1.5 ml centrifuge tube. The drop of mRNA is then drawn into the micropipette, taking care to avoid air bubbles. When the entire drop is drawn, ejecting one unit of mRNA should see a drop of mRNA rapidly form at or drop from the end of the micropipette.

4. Transfer ~20 oocytes into a 35 mm petri dish filled with OR2 and containing the plastic disc with slots or the wire mesh (Fig. 1a).

5. Impale the oocyte as little as possible while definitely reaching the cytoplasm with the micropipette tip, eject RNA, and remove micropipette quickly but carefully to avoid damaging the oocyte. Another

unit of RNA can be ejected in the OR2 to remove debris from the micropipette tip. Various laboratories report impaling the oocyte at different parts (e.g. middle of the animal pole or vegetal pole), but this author has typically injected into the equator. Oocytes can be rolled over with the micropipette, using the left hand to move the dish (and thus the oocyte) and the right hand to move the micropipette.

6. Although micropipettes can be used again immediately for another mRNA/set of oocytes, it is strongly recommended to replace, in order to avoid both clogging of the micropipette tip with debris and contamination of different RNAs.

7. Oocytes are transferred to a dish of Leibovitz's L-15 medium and stored at 18 °C until experiments (see Note 5). Oocytes may be stored with continuous stirring, which avoids deformation of the oocytes caused by sitting in the same position for hours/days. If after one day the media seems dirty, oocytes should be transferred to new Leibovitz's L-15 medium.

3.2 Two electrode voltage clamp recordings

1. Silver wire electrodes (these are included with the electrode holders but can be fashioned in-house with silver wire) should be coated in a AgCl layer by cleaning and then immersing (only as far as is necessary for inserting in KCl within the micropipette) in bleach. Bleach should then be rinsed off with deionized water, and if necessary carefully dried with a lint-free wipe (dabbing, to avoid wiping off the AgCl layer).

2. The glass micropipettes should be ~1/3- or ~1/5-filled (depending on length of Ag/AgCl electrode) with a MicroFil needle and syringe with 3 M KCl. Care should be taken to ensure all solution is in the end of the micropipette that will impale the oocyte, away from the top of the micropipette, where it can lead to salt build-up in the electrode holders. The micropipettes are then inserted into the micropipette holders, where part of the AgCl-coated electrode is in KCl.

3. The resistance of the microelectrodes can be tested by dipping them into the ND96 that perfuses the recording chamber. The voltage (difference between one electrode and its reference, bath electrode) here should be zero, and by using the membrane test on the amplifier, a current pulse is

injected and the calculated resistance is read out, which should be 0.5 – 1 MΩ for two electrode voltage clamp.

4. A single oocyte is placed in the recording chamber with continuous perfusion of ND96 solution. The oocyte is impaled with the two microelectrodes, both perpendicular to the oocyte membrane and no deeper than is necessary for obvious electrical connection, visible via the voltage readout on the amplifier (often around -30 mV when inside the oocyte). A scenario perhaps best described as "symmetrical" is essential for the smooth flow of solution around the oocyte (Fig. 1b,c). If this is not the case, perfusion may be unequal on different sides of the oocyte (which is already the case with the sides of the oocyte facing and facing away from the source of perfusion), and more importantly, the imbalance can tear the oocyte apart (Fig. 1d). Again, various laboratories may differ in the preference of which parts of the oocyte are impaled with the two microelectrodes, but this author prefers inserting both microelectrodes in the less yolky animal pole.

5. With both microelectrodes inside the oocyte and regular ND96 perfusing the oocyte, the amplifier can be set to voltage clamp, and the display for the voltage electrode will show the membrane potential of the oocyte membrane, depending on the user's input, and for resting GluCls, the display for current should read zero. Steady perfusion is essential (see Note 6). For oocytes expressing Cl^--selective GluCls, the reversal potential (see 3.3.2) is likely to be around -20 mV. Therefore, and depending on expression levels, oocytes might have to be clamped at -80 mV for currents of "workable" amplitude.

6. For measurement of agonist sensitivity of expressed GluCls, glutamate is applied for ~20 s between ~1 min rest periods and at increasing concentrations (Fig. 2a) by switching between lines on the perfusion system, one containing ND96, another 1 µM agonist in ND96, another 3 µM agonist in ND96, etc. Peak current amplitude can later be plot against glutamate concentration (3.3.1). For measurement of ion permeability (a measure of ion selectivity), the current—voltage (IV) relationship in different charge carriers is measured and the reversal potential of glutamate-gated currents in external ND96 can be compared to that in ND96/gluconate and ND96/choline (see 3.3.2 and Fig. 2d).

Numerous pharmacological assessments of GluCls can be performed by altering the mixtures of compounds dissolved in ND96 in different tubes of the perfusion system. Two examples are shown in Fig. 2a-c, including a test for agonist effects of selected compounds and a test for sensitivity of glutamate-gated currents to block by an inhibitor. Using site-directed mutagenesis and recording from mutant GluCls, the contribution of specific amino acid residues to GluCl function can be assessed (3.3.3).

7. Voltage clamp is switched off, micropipettes are removed from the oocyte, and the oocyte is disposed of. The same micropipettes can be used for subsequent oocytes/experiments, but over time, debris builds up on the micropipette tip, altering its resistance, and the AgCl layer will be removed from the electrode, often visible by a white, instead of black/brown, electrode. Therefore, glass micropipettes must be replaced and electrodes re-chloridized over the course of a day's recording.

3.3 Data analysis and interpretation

1. The sensitivity of GluCls to glutamate, other agonists and pharmacological modulators is typically quantified with half-maximal effective or inhibitory concentrations, EC_{50} and IC_{50}. These are calculated after plotting current (or inhibition of current) in response to concentration of agonist (or inhibitor) and fitting the data points with nonlinear regression

$$y = min + \frac{(max - min)}{1 + 10^{(logEC_{50} - x) \times n_H}}$$

where *y* = current response, *min* = minimum current response, *max* = maximum current response, EC_{50} = half maximal effective agonist concentration, *x* = agonist concentration, and n_H = cooperativity coefficient, or "Hill slope". Minimum current response is likely to be zero, and maximum is often normalized to one for each oocyte, because current amplitude varies across oocytes depending on expression. In the averaged data in Fig. 2b, the current responses (*I*, on the y-axis) of oocytes expressing SmGluCl-2 from *S. mansoni* to both L-glutamate and D-glutamate are normalized to the maximum current response to L-glutamate. As D-glutamate current amplitude is similar to that of L-glutamate, the former is also considered a full agonist. However, the EC_{50} for L- and D-isomers are ~20 µM and

~1mM, respectively, suggesting that the apparent affinity of the the D-isomer is much lower than that of the L-isomer.

2. As implied by the name, GluCls are selective for Cl^- over cations such as Na^+, K^+ and Ca^{2+}. With two electrode voltage clamp, this ion selectivity can be measured by substituting external chloride ions for less permeant anions such as gluconate and measuring a change in reversal potential (E_{rev}) of glutamate-gated currents.

The direction of Cl^- across the membrane through open GluCls depends primarily on two forces. The energy acting on ions due to the membrane potential (voltage) can be described by $-EzF$, where E is the membrane potential, z is the valency of the ion, and F is Faraday's constant. The chemical or diffusion energy acting on ions due the difference in intracellular and extracellular concentrations can be described by $-RT\ln([ion]_{out}/[ion]_{in})$, where R is the universal gas constant and T is temperature in Kelvin. The downward currents (which by convention show movement of positive charge into the cell) in Fig. 2a and c were recorded at -70 mV and reflect outward flow of Cl^-: despite the high $[ion]_{out}$ (103.6 mM Cl^- in ND96), Cl^- flows out due to the overwhelming electrical force (repulsion from inside the cell). Somewhere around E = -20 mV, however, $-EzF = -RT\ln([ion]_{out}/[ion]_{in})$. This is the reversal potential (E_{rev}) of glutamate-gated currents (x-intercept in Fig. 2d). Substituting extracellular gluconate for Cl^- (Na gluconate for NaCl) shifts E_{rev} to much more positive potentials, as the chemical energy forcing Cl^- inward is lessened and the electrical energy forcing Cl^- outward dominates (Fig. 2d). In contrast, substituting extracellular choline for Na^+ (choline Cl for NaCl) has no effect on E_{rev} as only impermeant ions have been affected [2].

3. In characterizing the molecular basis of GluCl function, wild-type (WT) glutamate sensitivity (or drug sensitivity, ion selectivity, etc) is compared to that of mutants containing single amino acid substitutions. Such experiments can be used to identify drug binding sites by identifying residues whose mutation most significantly affects drug sensitivity (but see Note 7). Mutations can then be "mapped" onto high-resolution structural (e.g. X-ray crystallographic) data of GluCls, if available, or onto homology models, if not [5].

4. Notes

1. If using an older digitizer, a computer/tower containing PCI or SCSI cards—and importantly, the size to accommodate them—is often necessary. This is not the case with modern digitizers that connect via USB.

2. For electrophysiological recordings from neurons or HEK cells, isolation of the set-up from mechanical vibrations and electrical noise is crucial. With larger, more robust oocytes, although a vibration control table (e.g. Cleanbench Laboratory Table from TMC) and a Faraday cage (eg. FAR01 from Thorlabs, Inc) may be required in some circumstances, a stable laboratory bench will often suffice, especially with a simple, steel base plate described above.

3. The stereomicroscope required for oocytes is generally simple, but when purchasing, a practical working distance (in which microelectrodes must be easily maneuvered) should be considered. This will be listed on the supplier's website, alongside combinations of objectives and eyepieces.

4. Traditionally, *X. laevis* is maintained in an animal facility on site and lobes of ovarian tissue are surgically removed, as described elsewhere [6,7]. Briefly, the frog is anaesthetized, lobes of ovarian tissue are removed with forceps via a ~1 cm abdominal incision (away from the midline), the wound is closed with three to five absorbable sutures, first internally, in the body wall, and secondly externally in skin, and the frog is left to recover in shallow water ensuring that the head is not submersed. Tissue is then manually separated into clumps of five to ten oocytes with forceps or tweezers, and outer follicular tissue is enzymatically removed by shaking incubation in OR2 containing 1 mg/ml collagenase. Some experimentation with collagenase concentration, shaking speed, incubation time, and temperature (e.g. room temperature vs 37 °C) will ensure optimal defolliculation. Collagenase is removed by several washes in OR2 before storing oocytes at 18 °C until use. Most descriptions of oocyte culture suggest storage in 35 mm Petri dishes. Oocytes may stick to plastic dishes (and subsequently rip), and therefore some trial and error of various plastic dishes, if not glass dishes or agarose coating may be required.

5. Functional surface expression of GluCls—or any receptor/channel—differs across clones, batches of oocytes, and laboratories. In this author's hands, the injection of 10 ng mRNA of the *Haemonchus contortus* AVR-14B GluCl yielded ~5 µA currents one day after injection, whereas the injection of 10 ng mRNA of *S. mansoni* SmGluCl-2 (from the same plasmid vector and transcription kit) yielded miniscule currents after one day, but >2 µA currents after three days. High-expressing constructs may require the injection of as little as 0.4 ng mRNA, whereas other constructs may require 40 ng. The injection of mRNA in a volume of 30-40 nl ensures reasonably consistent volume from oocyte to oocyte. The injection of 50 nl and higher is possible but likely to stress the oocyte (visibly). The expression of heteromeric GluCls is achieved by the mixing of different mRNAs shortly before injection [2]. Some channels (e.g. mutants) are constitutively active, leading to a leaky membrane and unhealthy oocytes. In this case, the addition of a channel blocker (e.g. picrotoxin) to the Leibovitz's L-15 medium may prevent the decline in oocyte health.

6. The rate of perfusion and the level of solution in the recording chamber, or the consistency thereof, is crucial for repeatable results. Most laboratories seem to use perfusion rates of one to two ml/minute. However, equally important to solution entering the recording chamber is solution leaving the chamber: inconsistent vacuum strength will see the level of solution rise and fall. This leads to numerous problems, including changes in the connection with bath electrodes, turbulent flow of perfusion solutions/drugs, and movement of the oocyte, which signals the rapid end to an experiment! Care should be taken ensure consistent perfusion and vacuum before starting experiments, e.g. by trimming/adjusting a small piece of tubing where the solution meets the vacuum/waste (Fig. 1b) and by checking tubes/connections in the waste collection system for leaks.

7. Shifts in EC_{50} must be interpreted carefully, as mutations can affect EC_{50} via numerous mechanisms, from ligand recognition, to altered channel gating (open—closed channel equilibrium), to desensitization (channel closure in the continued presence of agonist). This is famously discussed by Colquhoun [8], and although the interpretation of mutagenesis/two electrode voltage clamp data can

be aided by homology modeling [5], such questions are best addressed by measuring the kinetics of individual GluCls with more time-consuming patch clamp electrophysiology [9].

5. References

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Figure Captions

Fig. 1 Oocytes during injection of RNA and during two electrode voltage clamp experiments. (**a**) A plastic disc (grey) containing linear groves placed inside a 35 mm dish (white) provides a convenient base for injecting and even maneuvering oocytes during injection of RNA. (**b**) Side view of an oocyte during two electrode voltage clamp experiment (arrows indicate direction of perfusion). (**c**) Good and (**d**) poor impalement of the oocyte, viewed from above, in which uneven perfusion is likely to rip the oocyte from the microelectrodes.

Fig. 2 Characterization of GluCl function with two electrode voltage clamp. (**a**) Currents activated by application of agonists to oocytes expressing *S. mansoni* GluCl SmGluCl-2 and plot of current amplitude against agonist concentration. (**b**) Currents activated by application of glutamate alone or in the presence of thymol. (**c**) Glutamate-gated currents in the absence and presence of thymol. (**d**) Current amplitude (*I*) plot against membrane potential (*V*) in experiments with regular ND96 (NaCl) and ND96/gluconate.

Figure 1

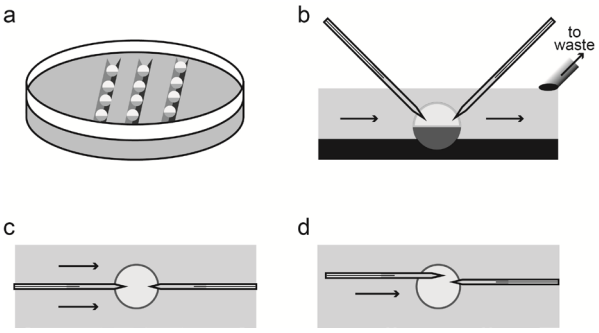

Figure 2