Low-Resistance silver bromide electrodes for recording fast ion channel kinetics under voltage clamp conditions

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ABSTRACT

Background: Two-electrode voltage clamp is a widely used technique for studying ionic currents. However, fast activation kinetics of ion channels are disguised by the capacitive transient during voltage clamp of Xenopus oocytes. The limiting factors of clamp performance include, but are not limited to, amplifier gain, membrane capacitance, and micropipette resistance. Previous work has focused on increasing amplifier gain (e.g.; high performing two-electrode amplifiers) or reducing the membrane capacitance (e.g.; the cut-open technique).

New method: The use of an Ag-AgBr electrode with saturated KBr solution to reduce micropipette resistance.

Results: The conductivity of 4 M KBr was 37 % higher compared to 3 M KCl and the micropipette resistance was reduced by 19 % when 4 M KBr was used, compared to the standard 3 M KCl solution. Micropipette resistances correlated positively with capacitive transient durations. Neither the current-voltage relationship of the voltage-gated sodium channel, Nav1.7, nor Xenopus oocyte stability were affected by bromide ions.

Comparison with existing methods: The de facto standard for two-electrode voltage clamp is 3 M KCl and Ag-AgCl electrodes, which are associated unnecessarily high micropipette resistance. Elsewise, cut-open voltage clamp techniques are technically demanding and require manipulation of the intracellular environment.

Conclusions: The use of an Ag-AgBr electrode with saturated KBr as micropipette solution reduces the capacitive transient in two-electrode voltage clamp recordings. Moreover, the exchange of chloride against bromide ions does not seem to affect oocyte physiology and ion channel kinetics.

1. Introduction

Voltage clamp electrophysiology recordings require rapid and precise control of the membrane potential. In most cases, the cellular capacitance is small, e.g., in neurons and mammalian cells. However, an exceptional case of high membrane capacitance is observed for the large *Xenopus laevis* oocytes when investigated using two-electrode voltage clamp (TEVC)(Guan et al., 2013). This heterologous expression system facilitates temporally resolved investigations of voltage-gated ion channels (Cota and Stefani, 1989; Llano and Bezanilla, 1980), ligand-gated ion channels (Corradi et al., 2015; Feng et al., 2002; Zwart et al., 2014), and G-protein coupled receptors (GPCR) (Sahlholm et al., 2014, 2016). By utilizing micropipettes with sub-micrometer tip diameters, a balance is struck between physical cell perturbation upon impalement and a swift current injection. However, for selected TEVC applications, the capacitive transient, evoked by cell membrane charging, may interfere with fast activation and/or inactivation kinetics of the voltage-gated ion channels to be investigated. To counteract the issues of capacitive transients, several methods have been developed, e.g. the cut-open voltage clamp (COVC) technique, which decreases the membrane area by enzymatic lipolysis that converts the intracellular compartment to an accessible compartment (Taglialatela et al., 1992). Although successful for investigating voltage-gated sodium (Nav) channels (Rojas et al., 1999; Smith et al., 1998) and gating currents (Perozo et al., 1994; Taglialatela and Stefani, 1993), COVC poses numerous technical challenges and implies manipulation of the intracellular milieu. In all, this motivated a search for alternative methods of decreasing the capacitive transient in conventional TEVC recordings and thereby facilitating the analysis of fast voltage-gated processes.

The TEVC circuit can, by ignoring complex electrochemical phenomena such as the influence of tip potential and non-ideal geometry, be reduced to a voltage follower, a voltage clamp operational amplifier, and a resistor-capacitance (RC) circuit, representing the cell and a number of resistors (Fig. 1). The decay time constant $\tau$, and the

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duration of the capacitive transient of the oocyte clamped in the TEVC is mainly dependent on three factors: i) the clamp gain $A$, ii) the membrane capacitance $C_m$, and iii) the resistance in the current-delivering micro-electrode $R_s$ according to the following relation:

$$\tau_C \approx \frac{R_s C_m}{A}$$

The clamp gain is dependent on the clamp apparatus used and the membrane capacitance on the endogenous properties of the oocytes. For *X. laevis* oocytes, the large diameter of approximately 1 mm provides convenient microscopic control of the micropipettes and solutions in relation to the oocyte. However, the membrane capacitance is large compared to small cells such as neurons. In contrast to clamp gain and membrane capacitance, the micropipette resistance depends on the tip diameter and the conductivity of the fluid. The tip diameter is precisely controlled during pipette pulling, although an increased diameter, with a corresponding decrease in resistance, might cause cell instability due to the trauma caused by the micropipette. Thus, assuming an optimal micropipette diameter, utilization of micropipette solution with high conductivity was hypothesized to decrease the capacitive transient decay time constant and thereby partly reduce the time course of the initial capacitive artefacts recorded in TEVC experiments.

Numerous TEVC protocols support the use of 3 M KCl as micropipette fluid (Dascal, 2001; Guan et al., 2013; Papke and Stokes, 2010), which is a natural extension of the Ag-AgCl electrode. The results presented here demonstrate that an alternative micropipette solution may reduce the micropipette resistance and shorten the membrane capacitive transient. This would allow for more accurate recordings of fast activation kinetics of voltage-gated ion channels using the TEVC technique. Moreover, the use of an Ag-AgBr electrode and KBr micropipette fluid is demonstrated not to interfere with oocyte stability or voltage-gated sodium 7 (Nav1.7) channel currents.

2. Materials and methods

2.1. Ionic conductivity measurements

Salts were weighted with 1 mg precision and dissolved in distilled water at room temperature (22 °C). Solubility titrations were performed using pipettes with 5 μL precision and conductivities were measured in 4–10 mL of solution using an Orion 135A Conductivity meter (ThermoFisher, MA) with automatic temperature compensation.

2.2. Micropipette preparation

GC150-7.5 borosilicate glass capillaries of 75 mm length, 1.5 mm outer and 0.86 mm inner diameter (Warner Instruments, CT), were pulled using the vertical Narishige PP-83 pipette puller (Narishige, Japan) to produce micropipettes. Heaters no. 1 and no. 2 were set to 13.50 and 9.36 respectively. Pipette tips and bodies were filled by syringe aspiration and injection of solution. The AgCl and AgBr electrodes were prepared by briefly applying a 9 V potential over the 0.3 mm Ag wire immersed into 3 M KCl and 4 M KBr solutions, respectively. The presence of AgCl and AgBr coatings was determined by inspection of the characteristic pale grey and yellow colors, respectively.

2.3. Oocyte isolation

Female African clawed toads (*X. laevis*) were anesthetized with MS-222 and oocytes were surgically extracted. The procedure was approved by the Swedish National Board for Laboratory Animals. The oocytes were incubated at 12°C in a solution containing 82.5 mM NaCl, 2 mM KCl, 24 mM NaHCO$_3$, 3.3 mM Ca(NO$_3$)$_2$, 4.1 mM CaCl$_2$, 8.2 mM MgSO$_4$, 0.15 mM HEPES and 2.5 mM sodium pyruvate, 25 μg/mL penicillin and 25 μg/mL streptomycin, adjusted to pH 7.6 with NaOH (Sigma, MO). For incubation buffers containing 10 %, 50 % and 100 % bromide, all chloride salts were substituted for the equimolar bromide salts. In subsequent experiments, oocytes were injected with 50 nl of 0.6 ng of RNA encoding Nav1.7 using the NanoJect II microinjector (Drummond Scientific, PA) (see Zeberg et al., 2020). The injected oocytes were incubated at 12°C for six days.

2.4. Electrophysiology

A 20 μL chamber, with the oocyte centered, was perfused with a bath solution (containing 88 mM NaCl, 1 mM KCl, 0.8 mM MgCl$_2$, 0.4 mM CaCl$_2$ and 15 mM HEPES, adjusted to pH = 7.4 with NaOH). Single oocyte two-electrode voltage clamp experiments were performed using the Dagan CA-1 setup (Minneapolis, MN). For Nav1.7 channel recordings, the semi-automatic, high-throughput, OpusXpress voltage clamp robot (Molecular Devices, CA) was utilized (Papke and Stokes, 2010), with the bath solution maintained at a reduced temperature (10°C). Data points were sampled with a frequency of 20 kHz using Molecular Devices software (pClamp 8.2). Uninjected oocytes were stepped to +30 and +60 mV from a holding potential of -80 mV. The capacitive transient was optimized during 3–6 trial runs by series resistance and capacitive transient compensations. Thereafter, measurements with the shortest capacitive transients were analyzed. Oocytes expressing Nav1.7 were held at -100 mV and voltage-stepped to +40 mV, using pulse increments of 10 mV.

2.5. Data analysis

Statistical analysis was performed using GraphPad 6 (Prism Software). Data are shown as mean ± SEM. Data normality was tested using the Anderson-Darling, Shapiro-Wilk, and D’Agostino-Pearson normality tests. Unpaired t-tests were used to compare micropipette resistances. Correlation was assessed using Spearman’s correlation test. $p < 0.05$ was considered significant. One-way ANOVA was used to compare resting membrane potentials. Solubility limits were rendered using Matlab software (MathWorks, MA).

3. Results

3.1. Determination of ionic conductivities

Saturated solutions of NaCl (4.5 M), NaBr (6 M), KCl (3.75 M) and KBr (4 M) were prepared by fully dissolving the salts in a minimal volume of distilled water. The conductivities increased with the...
conductivity was increased by 37.4% compared to 3 M KCl. None of the KCl/2.5 M NaBr increased the conductivity by 12.3% (714 mS). How- ever, the saturated KCl solution (3.75 M) alone increased the conduc-
tivity by 16.5% (741 mS) compared to 3 M KCl. For NaCl and KBr, similar results were noticed as combinations potentiated the conduc-
tivity at 5 M (see Fig. 2A). The findings support, and extend on, previously reported conductivity curves (Dobos, 1975; Lange and Dean, 1973; Robinson and Stokes, 2012; Weast, 1974). Moreover, the salt pairs NaBr/KCl and NaCl/KBr were dissolved in parallel, to derive a maximal solubility limit at which the ionic conductivities were measured (see Fig. 2B and C).

The results indicated that 3 M KCl, commonly utilized in TEVC micropipettes (Dascal, 2001; Guan et al., 2013; Papke and Stokes, 2010), has a conductivity of 636 mS (see Fig. 2A). A combination of 2.5 M KCl/2.5 M NaBr increased the conductivity by 12.3% (714 mS). However, the saturated KCl solution (3.75 M) alone increased the conductivity by 16.5% (741 mS) compared to 3 M KCl. For NaCl and KBr, similar results were noticed as combinations potentiated the conductivity, e.g. 2.5 M KBr/2.5 M NaCl measured to 699 mS. For 4 M KBr, the conductivity was increased by 37.4% compared to 3 M KCl. None of the conductivities at the maximum solubility limit of salt combinations exceeded the conductivity of the highest concentration of KCl or KBr.

3.2. Saturated KBr micropipettes in two-electrode voltage clamp recordings

To evaluate the effect of the ionic composition of the pipette solution on the micropipette resistances, measurements were performed by immersion into a TEVC chamber filled with the bath solution. With 4 M KBr micropipettes, the resistance was reduced significantly by 85 kΩ or 19%, as compared to 3 M KCl (see Fig. 3A). Next, the relation between micropipette resistance and capacitive transient duration was assessed in native X. laevis oocytes. The oocytes were voltage clamped at -80 mV and stepped up to +30 or +60 mV (Fig. 3B, left and right panels, respectively). The resulting currents indicated that a low resistance was indeed related to a short capacitive transient.

To investigate the feasibility of the Ag/AgBr electrode pair with 4 M KBr micropipette fluid in the context of ion channel TEVC recordings, X. laevis oocytes were injected with the Nav1.7 channel RNA transcript (SCN9A gene). The recordings from representative oocytes, using 4 M KBr/Ag- AgBr electrodes and 3 M KCl/Ag-AgCl electrodes, are depicted in Fig. 3C (left and middle panels). The currents were verified as resulting from Nav channel opening by 60 s application of tetrodotoxin (TTX); application of 1 μM TTX abolished the inward currents (Fig. 3C, right panel). The current-voltage relationship of the Nav1.7 channel was similar when using 4 M KBr and Ag-AgBr electrodes, and in agreement with previous descriptions (Fig. 3D)(Estacion and Waxman, 2017; Xiao et al., 2010; Zeberg et al., 2020). The physiological consequences following substitution of KCl for KBr in the incubation solution were evaluated by X. laevis oocyte survival and resting membrane potential. Native oocytes were incubated in four cell culture solutions with 0%, 10%, 50% and 100% substitution of chloride salts for the corresponding bromide salts and cell survival was assessed by microscope daily, with identical survival between the groups (not shown). Furthermore, results suggested that substitution of all extracellular chloride ions for bromide ions did not affect oocyte resting membrane potentials after six days of incubation (Fig. 3E).

4. Discussion

This investigation demonstrates that saturated KBr solution and Ag- AgBr micropipette electrodes reduce the resistance and thereby the capacitive transient in TEVC recordings, compared to standard KCl/Ag- AgCl counterparts. Moreover, the impact of bromide ions on Nav channel recordings and cell stability is shown to be very limited.

4.1. Implications of bromide ions in micropipettes

From an electrophysiological perspective, the Ag/AgBr-reaction differs in potential compared to the traditionally used Ag/AgCl- reaction. Thus, the Ag/AgBr-electrode and the 4 M KBr micropipette fluid confers an experimental electrode offset around -200 mV for the current-injecting electrode. However, the current-injecting electrode does not require a specific offset, as opposed to the extracellular and intracellular voltage-recording electrodes. Although an introduction of bromide ions into the oocyte may occur during TEVC with Ag-AgBr electrodes, due to diffusive processes between the micropipette tip and the cytosol, the ions are not expected to cause any significant disturbances, neither of the ion channel functioning nor the normal physiology of the oocytes. Although this was exemplified experimentally by the equal survival and unaffected resting membrane potential of cells incubated in solutions with varying degrees of bromide-for-chloride substitution, other physiological and electrophysiological consequences of bromide ions cannot be completely excluded.

4.2. Geometrical aspects of resistivity measurements

Previously, the micropipette resistance has been described to depend on the conductivity of the pipette solution, and the diameter and tip angle of the micropipette (Snell, 1969). This relationship is addressed theoretically in Appendix A.
4.3. Potential applications and future outlooks

The capacitive transient is inversely proportional to the resistance of the current-injecting micropipette. Thus, a reduction of the micropipette resistance allows for use of either a smaller diameter micropipette without affecting the capacitive transient duration, or a reduction of capacitive transient duration at any given micropipette diameter. The former option may prove relevant when GPCR signaling is investigated using a G protein-coupled inwardly-rectifying potassium channel assay, in order to reduce impalement trauma to the cell, which potentially could affect cell stability during long-timescale voltage clamp recordings (10–15 min), or when measuring very small currents (Ågren et al., 2018; Sahlholm et al., 2016). The latter alternative is suitable when investigating fast voltage-gated processes at a millisecond time scale, as seen in the Nav channel family (see Results). Future improvements for shortening the capacitive current time course could involve introduction of a second current-injecting micropipette, theoretically halving the capacitive transient duration.

4.4. Conclusions

This report shows that micropipettes containing an Ag-AgBr electrode and 4 M KBr solution reduce the resistance and thereby the capacitive transient duration. This is particularly useful when investigating voltage-gated ion channels with fast activation and/or inactivation processes; e.g., Nav channels.

Fig. 3. Application of saturated potassium bromide micropipettes in two-electrode voltage clamp recordings. A) Bath resistances of micropipettes containing 3 M KCl and 4 M KBr. 4 M KBr decreased the micropipette resistance (-0.085 ± 0.033 MΩ, p = 0.023, n = 7 per group, unpaired t-test). B) Capacitive transient durations, measured at the baseline, correlated with micropipette resistance following 20 ms depolarizations from the holding potential, -80 mV, to +30 (Spearman’s r = 0.7350, p = 0.045, n = 8; left panel) and +60 mV (Spearman’s r = 0.7928, p = 0.0397, n = 7; right panel). Recordings were performed in un.injected oocytes. C) Recording of Nav1.7 fast kinetics using Ag/AgBr electrodes with 4 M KBr (left panel), 3 M KCl (middle panel) and following addition of 1 μM tetrodotoxin (TTX; right panel). The traces represent the holding potential, -100 mV (black), depolarizations to -20 mV (red), -10 mV (green), 0 mV (blue), 10 mV (pink), and 30 mV (orange). The current amplitude at steady-state, 150 ms into the respective voltage-step, was considered as baseline and was subtracted from each trace. Voltage-steps were 385 ms long and the interpulse interval was 5 s. The holding potential was -100 mV. D) I–V relationships from C), between -100 and +40 mV. Number of oocytes: 4 M KBr, n = 4; 3 M KCl, n = 7, TTX; n = 3. E) Oocyte resting membrane potential (RMP) at 6 days after extraction, when incubated in different concentrations of chloride and bromide ions (n = 20 per group). The RMPs did not differ significantly (one-way ANOVA, comparison with 0% Br⁻) between oocytes incubated in 0-100% bromide ions. Normality of data in A) and E) was confirmed using the Anderson-Darling, Shapiro-Wilk, and D’Agostino-Pearson normality tests (p > 0.05). * indicates p < 0.05, unpaired t-test.
CRediT authorship contribution statement

Richard Ågren: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. Hugo Zeberg: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jneumeth.2020.108984.

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