Journal of Visualized Experiments

Measurement of ion concentration in the unstirred boundary layer with open patch-clamp pipette: implications in control of ion channels by fluid flow

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58228R1
Full Title:	Measurement of ion concentration in the unstirred boundary layer with open patch-clamp pipette: implications in control of ion channels by fluid flow
Keywords:	Fluid flow; Shear force; Unstirred layer; Patch clamp; Ag/AgCl reference electrode; Liquid/metal junction potential; convection; ion channel
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Biomedical Research Center, 120, Neungdong-ro, Gwangjin-gu, Seoul 05029 KOREA)

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2018-09-07

Vineeta Bajaj,
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Dear editor Vineeta Bajaj,

Thank you very much for reviewing our manuscript. We appreciate the helpful and constructive comments from the editor and the reviewers. We have responded to all comments raised by the editor and the reviewers and revised the manuscript accordingly. The title of the manuscript has been changed to “Measurement of ion concentration in the unstirred boundary layer with open patch-clamp pipette – implications in control of ion channels by fluid flow” from “Protocols for fluid shear force-regulation of ion channels in patch clamp recordings.” We hope that JOVE will find this revised manuscript acceptable.

Yours sincerely,

Young Min Bae

TITLE:

Measurement of Ion Concentration in the Unstirred Boundary Layer with Open Patch-Clamp Pipette: Implications in Control of Ion Channels by Fluid Flow

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KEYWORDS:

Fluid flow, shear force, unstirred layer, patch-clamp, Ag/AgCl reference electrode, liquid/metal junction potential, convection, ion channel

SUMMARY:

Mechanosensitive ion channels are often studied in terms of fluid flow/shear force sensitivity with patch-clamp recording. However, depending on the experimental protocol, the outcome on fluid flow-regulations of ion channels can be erroneous. Here, we provide methods for preventing and correcting such errors with a theoretical basis.

ABSTRACT:

Fluid flow is an important environmental stimulus that controls many physiological and pathological processes, such as fluid flow-induced vasodilation. Although the molecular mechanisms for the biological responses to fluid flow/shear force are not fully understood, fluid flow-mediated regulation of ion channel gating may contribute critically. Therefore, fluid flow/shear force sensitivity of ion channels has been studied using the patch-clamp technique. However, depending on the experimental protocol, the outcomes and interpretation of data can be erroneous. Here, we present experimental and theoretical evidence for fluid flow-

related errors and provide methods for estimating, preventing, and correcting these errors. Changes in junction potential between the Ag/AgCl reference electrode and bathing fluid were measured with an open pipette filled with 3 M KCl. Fluid flow could then shift the liquid/metal junction potential to approximately 7 mV. Conversely, by measuring the voltage shift induced by fluid flow, we estimated the ion concentration in the unstirred boundary layer. In the static condition, the real ion concentrations adjacent to the Ag/AgCl reference electrode or ion channel inlet at the cell-membrane surface can reach as low as approximately 30% of that in the flow condition. Placing an agarose 3 M KCl bridge between the bathing fluid and reference electrode may have prevented this problem of junction potential shifting. However, the unstirred layer effect adjacent to the cell membrane surface could not be fixed in this way. Here, we provide a method for measuring real ion concentrations in the unstirred boundary layer with an open patch-clamp pipette, emphasizing the importance of using an agarose salt-bridge while studying fluid flow-induced regulation of ion currents. Therefore, this novel approach, which takes into consideration the real concentrations of ions in the unstirred boundary layer, may provide useful insight on the experimental design and data interpretation related to fluid shear stress regulation of ion channels.

INTRODUCTION:

Fluid flow is an important environmental cue that controls many physiological and pathological processes such as fluid flow-induced vasodilation and fluid shear force-dependent vascular remodeling and development[1-5]. Although the molecular mechanisms for the biological responses to fluid flow shear force are not fully understood, it is believed that fluid flow-mediated regulation of ion channel gating may critically contribute to fluid flow-induced responses[5-8]. For example, activation of the endothelial inward rectifier Kir2.1 and Ca^{2+}-activated K^+ ($K_{Ca}2.3$, KCNN3) channels after Ca^{2+} influx by fluid flow has been suggested to contribute to fluid flow-induced vasodilation[6-8]. Therefore, many ion channels, especially mechanically-activated or -inhibited channels, have been studied in terms of fluid flow/shear force sensitivity with the patch-clamp technique[6,9-11]. However, depending on the experimental protocol performed during patch-clamp recording, outcomes and interpretation of the data on fluid flow-regulations of ion channels can be erroneous[10,11].

One source of fluid flow-induced artifacts in patch-clamp recording is from the junction potential between the bath fluid and Ag/AgCl reference electrode[11]. It is generally believed that the liquid/metal junction potential between the bathing fluid and Ag/AgCl electrode is constant as the Cl^- concentration of the bathing fluid is kept constant, considering the chemical response between the bathing solution and Ag/AgCl electrode to be:

$$Ag + Cl^- \leftrightarrow AgCl + \text{electron } (e^-) \quad (\textbf{Equation 1})$$

However, in a case where the overall electrochemical reaction between the bathing solution and Ag/AgCl reference electrode (**Equation 1**) proceeds from left to right, the Cl^- concentration of the bathing fluid adjacent to the Ag/AgCl reference electrode (unstirred boundary layer[12-15]) may be much lower than that in the bulk of bathing solution, unless enough convectional transport is ensured. Using an old or non-ideal Ag/AgCl electrode with inadequate chlorination

of Ag may increase such a risk. This fluid flow-related artifact at the reference electrode, in fact, can be excluded by simply placing a conventional agarose-salt bridge between the bathing fluid and reference electrode, since the artifact is based on alterations in real Cl^- concentration adjacent to the Ag/AgCl electrode[11]. The protocol presented in this study describes how to prevent the flow-related junction potential changes and measure real ion concentrations in the unstirred boundary layer.

After placing an agarose KCl bridge between the bathing fluid and Ag/AgCl reference electrode, there is another crucial factor that should be considered: just as the reference Ag/AgCl electrode acts like a Cl^- electrode, the ion channels also can function like an ion-selective electrode. The situation of an unstirred boundary layer between the bathing fluid and Ag/AgCl reference electrode arises during the movement of ions between the extracellular and intracellular solutions through the membrane ion channels. This implies that caution should be used when interpreting the regulation of ion channels by fluid flow. As discussed in our previous study[11], the movement of ions through a solution in which an electrochemical gradient is present can occur *via* three distinct mechanisms: diffusion, migration, and convection, where diffusion is the movement induced by concentration gradient, migration is the movement driven by electrical gradient, and convection is the movement through fluid-flow. Among these three transport mechanisms, convection mode contributes most to the movement of ions[11] (> 1,000 times greater than diffusion or migration under usual patch-clamp settings). This forms the theoretical basis of why junction potential between the bathing fluid and Ag/AgCl reference electrode can very under different static and fluid-flow conditions[11].

As per the hypothesis proposed above, some facilitatory effects of fluid flow on the ion channel current may be inferred from the convective restoration of real ion concentrations adjacent to the channel inlet at the membrane surface (unstirred boundary layer)[10]. In this case, the fluid flow-induced effects on ion channel currents have simply arisen from electrochemical events, not from the regulation of ion channel gating. A similar idea was previously suggested by Barry and colleagues[12-15] based on rigorous theoretical considerations and experimental evidence, also known as the unstirred layer or transport number effect. If some ion channels have sufficient single channel conductance and long enough open-time to provide sufficient transport rates through the channels (a faster transport rate in the membrane than in the unstirred membrane surface), a boundary layer effect may arise. Thus, the convection-dependent transport can contribute to the eventual fluid-flow-induced facilitations of ion current[10,12-15].

In this study, we emphasize the importance of using an agar or agarose salt-bridge while studying fluid-flow-induced regulation of ion currents. We also provide a method for measuring real ion concentrations in the unstirred boundary layer adjacent to the Ag/AgCl reference electrode and membrane ion channels. Furthermore, the theoretical interpretation of fluid flow-induced modulation of ion channel currents (*i.e.,* convection hypothesis or unstirred layer transport number effect) can provide valuable insights for designing and interpreting studies on the shear force-regulation of ion channels. According to the unstirred boundary layer transport number effect, we predict that ion channel currents through all types of membrane ion

channels can be facilitated by fluid flow, independent of their biological sensitivity to fluid flow shear force, but only if the ion channels have sufficient single channel conductance and long open-time. Higher ion channel current densities may increase the unstirred boundary layer effect at the cell membrane surface.

PROTOCOL:

All experiments were performed in accordance with the institutional guidelines of Konkuk University.

1. Agarose Salt Bridges Between the Bath Solution and Ag/AgCl Reference Electrode

Note: Agarose 3M KCl salt bridges are produced as previously described[12] with minor variations.

1.1. Formation of bridges

1.1.1. Bend the fire glass capillary tubes to form a U-shape as appropriate. The inner diameter of the capillaries should be large enough for reducing series resistance when recording large ion currents. Tubes with an inner diameter of 2-5 mm are usually acceptable.

1.2. Preparation of agarose 3 M KCl solution

1.2.1. Prepare 200 mL of 3 M KCl solution (1 M or 2 M is also acceptable).

1.2.2. Weigh 10 g of agarose.

1.2.3. Dissolve the agarose in 200 mL of KCl (*i.e.,* 5% agarose) on a hot plate between 90 and 100 °C.

1.3. Loading the bridges with 3 M KCl agarose

1.3.1. For easy loading, immerse the U-shaped glass bridges in the agarose-KCl solution.

Note: It is easy to dig out the glass bridges if the agarose-KCl solution is contained in a shallow and broad container.

1.3.2. Keep them overnight at room temperature (RT) for the agarose to set and harden.

1.3.3. Carefully dig out the agarose-KCl-loaded glass bridges from the set/hardened agarose-salt.

1.4. Storing the bridges

1.4.1. Prepare enough volume (*i.e.,* 500 mL) of the 3 M KCl solution in a wide-necked bottle.

1.4.2. Store the prepared agarose-salt bridges in the bottle in a refrigerator.

2. Application of Fluid Flow Shear Force to Cells in a Patch-Clamping Chamber

Note: A schematic diagram of the patch-clamp experimental set-up is shown in **Figure 1**.

2.1. Place a container loaded with bathing solution (volume and height should already be measured) above the patch-clamp chamber.

2.2. Fill the patch-clamp chamber with the bathing solution by suctioning the tube.

2.3. To stop the fluid flow, clip the tube at the container's side to block the fluid flow, then clip the tube at the suction side to stop the suction at the same time. This is the "stationary" control condition.

2.4. To apply fluid flow shear force, open both tubes on the container and suction sides at the same time.

2.5. Before or after applying the fluid flow shear force to the cell, measure the flow rate in mL/min.

2.6. Calculate the flow rate by measuring the decrease in fluid volume over a given time.

2.7. From the measured flow rate and geometry (structure) of the bathing chamber, the shear force applied to the cell by the fluid flow should be estimated (see discussion section).

2.8. Alternatively, to control the flow rate (for steps 2.3-2.6), use a perfusion pump. In this case, be careful to ensure a constant rather than a pulsatile flow.

3. Measuring Changes in Liquid-Metal Junction Potential by Fluid Flow Between Bath Solution and Ag/AgCl Reference Electrode (Figure 3A)

3.1. Use the Ag/AgCl electrode or pellet, which is available from the ready-made products, without the agarose salt bridge.

3.2. Prepare a normal physiological salt saline for the bathing chamber (*e.g.*, 143 mM NaCl, 5.4 mM KCl, 0.33 mM NaH_2PO_4, 5 mM HEPES, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11 mM D-glucose; pH adjusted to 7.4 with NaOH).

3.3. Place a patch pipette containing a 3 M KCl solution in the chamber to minimize the junction potential shift between the pipette and bathing solutions.

3.4. Fix the voltage-clamp amplifier to the current clamp mode ("I = 0" or "CC").

3.5. After nullifying the initial offset potential, measure changes in voltage induced by various flow rates.

3.6. To verify that the changes in voltage are liquid/metal junction potentials, re-examine the effect of fluid flow on the junction potential using the agarose-salt bridge between the bath solution and Ag/AgCl electrode.

4. Experimental Estimation of Real Cl^- Concentration in the Unstirred Layer Adjacent to Ag/AgCl Electrode Under Static Condition (Figure 3B)

4.1. From the results of step 3, draw the junction potential-flow rate relationships and estimate the maximal (saturating) value of junction potential shift by the supra-fluid flow rate.

4.2. Prepare solutions with various concentrations of Cl *(i.e.,* 50, 99, 147, 195, and 288 mM of NaCl).

4.3. By changing the Cl^- concentration in the bathing fluid, draw the junction potential-[Cl^-] relationship. Note that the fluid rate should be constant and sufficiently high (> 30 mL/min) to prevent the decrease of Cl^- concentration to that of the adjacent Ag/AgCl reference electrode.

4.4. From the two relationship curves, estimate the changes in Cl^- concentration from the measured junction potential shift.

REPRESENTATIVE RESULTS:

Whole cell voltage-dependent L-type Ca^{2+} channel ($VDCC_L$) currents were recorded in the enzymatically dispersed rat mesenteric arterial myocytes, as previously described[11]. The arterial myocytes were dialyzed with a Cs-rich pipette solution under the nystatin-perforated configuration with divalent cation-free bathing solution to facilitate the current flow through $VDCC_L$[11,16]. Brief depolarizing voltage ramps or voltage steps, at a holding potential of -70 mV, were applied to elicit the $VDCC_L$ currents. A representative current-voltage (*I*-V) relationship in $VDCC_L$ in the absence and presence of fluid flow (5 mL/min or approximately 0.004 m/s), recorded with an agarose KCl bridge, is shown in **Figure 2A**. Fluid flow slightly increased the $VDCC_L$ current in a voltage-independent manner. This facilitating effect of fluid flow on the $VDCC_L$ current is summarized in **Figure 2B**.

The voltage-independent facilitation of $VDCC_L$ current by fluid flow is a proper response of the $VDCC_L$ to the fluid or shear force. The 5 mL/min or approximately 0.004 m/s of fluid flow in the current experimental setup was estimated to represent approximately 0.1 dyn/cm^2 in terms of shear force (see discussion). However, when the Ag/AgCl reference electrode was directly linked to the bathing fluid without an agarose KCl bridge, the *I*-V relationship in the presence of fluid flow shifted to the right compared to that of the $VDCC_L$ currents under a static condition (**Figures 2C** and **2D**). This resulted in the inhibition of $VDCC_L$ current at negative voltages and facilitation of $VDCC_L$ current at more depolarized or positive potentials. This exemplifies the fluid flow-induced artifact in patch-clamp recording in which a voltage shift of the *I*-V relation

was not due to the modification of channel gating but was actually due to a junction potential shift between the bathing fluid and Ag/AgCl reference electrode[11]. Direct evidence for the fluid flow-induced junction potential shift is shown in **Figure 3**.

The junction potential shifts were measured according to step 3. The changes, due to fluid flow, were measured using an open pipette filled with 3 M KCl, as previously described[11]. With an open pipette filled with 3 M KCl, the junction potential between the pipette and bathing solutions could be minimized, and the potential changes due to fluid flow were primarily from the bathing solution and Ag/AgCl reference electrode. Without an agarose 3 M KCl bridge between the bathing fluid and Ag/AgCl reference electrode, fluid flow shifted the junction potential between the fluid and the Ag/AgCl electrode in a fluid flow rate-dependent manner (**Figure 3A**). The maximum junction potential change was extrapolated to be ~7 mV from the junction potential-fluid flow relationship (**Figure 3A**, bottom). In contrast, when the agarose 3 M KCl bridge was used, fluid flow did not alter the junction potential between the bathing fluid and reference electrode (summarized in the bottom graph of **Figure 3A**, bottom).

In order to measure concentration differences between the static and fluid flow conditions, in which enough convection modes of action are functional, we examined the effect of changing Cl^- concentrations on the bathing fluid-Ag/AgCl electrode junction potential according to step 4. Increasing the Cl^- concentration shifted the junction potential in a concentration-dependent manner (**Figure 3B**, top) just as fluid flow shifted the junction potential in a rate-dependent manner. Using a KCl agarose bridge, the junction potential was prevented from changing in a Cl^- concentration-dependent manner (**Figure 3C**), indicating that the junction potential change occurred between the bath solution and reference electrode, not between the bath and pipette solutions. The semi-log plot of the junction potential-$[Cl^-]$ relationship is shown in the lower panel of **Figure 3B**. According to the results in **Figure 3B**, the extrapolated maximal value of ~7 mV in junction potential shift (from **Figure 3A**) suggests that the Cl^- concentration adjacent to the Ag/AgCl reference electrode decreases to ~70% of the average concentration of the bulk bathing fluid when fluid flow is absent (**Figure 3B**, bottom).

In our previous study, Kir2.1 currents were reported to be facilitated by fluid flow by convectively restoring (increasing) $[K^+]$ at the channel inlet[10]. This idea stems from the phenomena occurring between the bathing fluid and Ag/AgCl electrode, as the Kir2.1 channel can function as a K^+ electrode just as the Ag/AgCl electrode functions as a Cl^- electrode. This idea is schematically illustrated in **Figures 4A** and **4B**. A representative example of fluid flow-induced facilitation of Kir2.1 currents is shown in **Figure 4C**. The Kir2.1 currents were elicited by a hyperpolarizing voltage step from a holding potential of 0 to -100 mV in rat basophilic leukemia (RBL) cells. Application of fluid flow (5 mL/min or 0.004 m/s) readily increased the Kir2.1 current (**Figure 4C**). This facilitation by fluid flow was previously suggested to be mediated not by cellular signaling but by the electrochemical effect of convective transportations of K^+ ions to the unstirred boundary layer[10].

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic showing setup of the bathing chamber for the fluid-flow regulation of ion channels in the patch-clamp recording. Lower panel is the side view (sagittal section) of the patch-clamp chamber. It summarizes the path of fluid flow and locations of a studied cell, electrodes, and inlet/outlet of the fluid. Because the fluid is continuously pumped out through outlet tube by suction, the height of fluid in the chamber is maintained at a relatively constant level. This figure has been modified from a previous publication[11].

Figure 2: Effects of fluid flow on L-type voltage-dependent Ca^{2+} channel ($VDCC_L$) currents with and without the agarose 3 M KCl bridge. $VDCC_L$ currents were recorded in the enzymatically dispersed rat mesenteric arterial myocytes with nystatin perforated patch-clamp recording. Normal tyrode physiological salt solution with 4.2 mM EDTA without divalent cations was used as the bathing solution[11]. The pipette solution contained CsCl, 140 mM; $MgCl_2$, 1 mM; HEPES, 5 mM; EGTA 0.05 mM; pH adjusted to 7.2 with CsOH. (A and B) With agarose 3M KCl- bridge. (A) A representative *I*-V relationship for the $VDCC_L$ current and the effects of fluid flow. (B) Summary of the fluid effects on the *I*-V relationship of $VDCC_L$ currents. (C and D) Without agarose 3M KCl bridge. (C) *I*-V relationships of the $VDCC_L$ currents. (D) Summarized *I*-V relationships of the peak $VDCC_L$ currents in the absence and presence of fluid flow. The shapes of voltage steps for eliciting $VDCC_L$ currents are shown in the figure inset. This figure has been modified from a previous publication[11].

Figure 3: Effects of fluid flow on liquid-metal junction potential between the bathing fluid and Ag/AgCl reference electrode and estimation of real Cl^- concentration in the unstirred layer adjacent to the reference electrode from the measured junction potential. (A) A representative tracing of junction potential changes due to various rates of fluid flow (upper panel). This figure has been modified from a previous publication[11]. The junction potential-fluid flow rate relationship (n = 5). (B) Upper panel: representative recording of junction potential changes due to various concentrations of NaCl solutions. Lower panel: the semi-log plot of the junction potential-[Cl^-] relationship (n = 5). The straight line in red represents the best fit by a modified Nernst-equation for equilibrium potential with a ten-fold slope of 49 mV. Owing to the finite selectivity of Na^+, compared to that of Cl^-, for generating the liquid/metal junction potential, the slope value of 49 mV, instead of 58 mV, produced the best fit in the junction potential-[Cl^-] relation at room temperature. The 49-mV slope indicates the Cl^- dependence (or selectivity) of the Ag/AgCl reference electrode > 95% over the other ion (in this case, Na^+), according to the Goldman-Hodgkin-Katz voltage equation. A shift of 7 mV at an Cl^- concentration of 150 mM indicates a decrease of ~30% in the Cl^- concentration. (C) A representative tracing of junction potential in various concentrations of NaCl solutions with a use of 3 M KCl agarose bridge (n = 3).

Figure 4: Schematic of the effects of convection model of fluid flow on the ion concentrations adjacent to the open channels during ion current flux. (A) Under static conditions with little convective transport of ions in the solution with electric field, the K^+ ion flux through K^+-selective ion channels can cause a decrease in K^+ concentrations in the microdomain adjacent to the channel inlet. (B) Fluid flow can convectively restore the decrease in K^+ concentration adjacent to the open channel inlet. (C) Effect of fluid flow on the inward rectifier Kir2.1 channel

currents. Fluid flow instantly increased the Kir2.1 currents. The shape of the voltage step is shown in the figure inset. The Kir2.1 currents were recorded using high K^+-bathing and -pipette solutions. Bathing solution: 148.4 mM KCl, 0.33 mM NaH_2PO_4, 5 mM HEPES, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11 mM D-glucose; pH adjusted to 7.4 with NaOH. Pipette solution: 135 mM KCl, 5 mM NaCl, 5 mM Mg-ATP, 10 mM HEPES, 5 mM ethyleneglycol-bis (2-aminoethyl)-N,N,N',N',-tetraacetic acid (EGTA), pH 7.2 (adjusted with KOH). Since RBL-2H3 cells are highly susceptible to hypo-osmotic swelling and consequent trigger of volume-activated Cl^- currents, 38 mM sucrose was added to the bathing solution to adjust for osmolarity and prevent cell swelling. Moreover, a Cl^- channel blocker [4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS, 30 µM)] was added to the pipette solution to eliminate any contamination by Cl^- currents. Panel C has been modified from a previous publication[10].

DISCUSSION:

In this study, we demonstrated a method to measure real Cl^- concentration in the unstirred layer adjacent to the Ag/AgCl reference electrode by determining the liquid-metal junction potential with an open patch-clamp pipette filled with a high KCl concentration. The change in Cl^- concentration in the boundary layer can result in a shift of junction potential when switching from static to fluid-flow conditions. Simply using an agarose KCl bridge between the reference electrode and bathing fluid can prevent the Cl^- concentration-related errors or artifacts during patch-clamp recording.

Besides emphasizing the importance of an agar or agarose salt bridge, another application of this method in estimating real ion concentration in the unstirred boundary layer is as follows. Because plasmalemmal ion channels can function as ion-selective electrodes (just as the Ag/AgCl electrode functions like a Cl^- electrode), the real ion concentration in the unstirred boundary layer adjacent to the channel inlet at the cell membrane surface can be different from the average concentration of the bulk fluid. This difference in ion concentration between the bulk fluid and unstirred layer adjacent to cell membrane is the real scenario under clinical settings and should be distinguished from the biological modulation of channel gating by fluid flow/shear force. Unfortunately, unlike the unstirred layer effect between the Ag/AgCl reference electrode and bathing fluid, we cannot fix the unstirred layer effect adjacent to the cell membrane surface when studying the regulation of ion channels by fluid flow/shear force.

However, considering the observation that real ion concentration in the unstirred layer is approximately 70% of that in bulk fluid (**Figure 3**), we can make some amendments in the experimental data to distinguish the biological modulation of ion channels from the "electrochemical phenomenon of unstirred layer effect". The real ion concentration in the unstirred layer at the cell membrane surface was expected to be approximately 70% of the average concentration of the bulk bathing solution in a recent study[10]. Since fluid flow restored the decreased ion concentration, it facilitated the Kir2.1 current independently of cellular signaling[10]. In our previous study, current density was considerably high (2.5 A/m^2) with a high extracellular K^+ concentration and high expression of Kir2.1 in RBL cells[10]. However, in the case of real cell membranes with various ion channel current density amplitudes, the unstirred layer effect at the cell membrane surface may depend greatly on the amplitude of ion channel

current density. Besides, this may cause some ion channel currents (especially those with relatively lower current densities) to be insensitive to fluid-flow regulation; although, the unstirred layer effect is regulated electrochemically and not biologically. Thus, this may affect the technique described here. Therefore, the possibility of developing a quantitative method that is adequate for correcting experimental results should be investigated in future studies.

In **Figure 3**, we observed that liquid-metal junction potential between the Ag/AgCl reference electrode and bathing fluid was greatly dependent on the condition of the Ag/AgCl electrode. In fact, when the Ag/AgCl electrode was perfect in condition, changes in junction potential due to fluid flow was minimal (data not shown). However, poor chlorination of the Ag/AgCl electrode caused a greater shift in the junction potential. Since the Ag/AgCl reference electrode is very susceptible to various external stimuli, such as ultraviolet light and oxidative stress, using an agar or agarose KCl bridge is always recommended. Although changes in junction potential by fluid flow between bathing fluid and the reference electrode is a potential source of error, we successfully estimated the real ion concentrations in the unstirred boundary layer by measuring the shift of junction potential under various fluid-flow rates (**Figures 3A** and **3B**).

The critical point in step 4 for preparing the standard curve for the estimation of real Cl^- concentration in the unstirred boundary layers from the shift of junction potential is that the standard curve should be recorded under a sufficent flow rate (30 mL/min in this experiment). Although this flow rate is very fast, in practical cases the faster the fluid, the smaller the concentration drop is at the boundary layers (**Figure 3**). In addition, the open pipette must be filled with high KCl, instead of a regular pipette solution, in order for a patch-clamp study to prevent the change in junction potential between a pipette and bathing solution.

The shear force in the patch-clamp setting can be estimated from the following relationship[11]:

$$\tau = (6\mu Q) / (bh^2) \quad \textbf{(Equation 2)}$$

Where: τ is the shear stress (N/cm^2); μ is the viscosity (0.001 N m/s^2 for water at 20°C); Q is the fluid flow rate (m^3/s); b is the chamber width (m); and h is the chamber height (m). When the fluid flow rate is 30 mL/min, the shear force in the patch-chamber shown in **Figure 1** is estimated to be ~0.75 dyn/cm^2 according to the above equation. This is a low shear force level compared to the physiological shear force; endothelial cells in blood vessels can be subjected to shear forces of up to 40 dyn/cm[18,19,21]. Therefore, provided that the ion channels are not sensitive to shear forces less than 0.75 dyn/cm^2, we can study the fluid flow/shear force sensitivity of ion channels after excluding the unstirred boundary layer effect by setting the control condition to 0.75 dyn/cm^2. However, some ion channels, including Kir2.1, seem to be sensitive to shear forces less than 0.75 dyn/cm[2-6].

The unstirred layer effect was originally suggested by Barry and colleagues[12-15]. Here, we provide a method to estimate real ion concentration in the unstirred layer by measuring changes in junction potential with open patch-clamp pipette. We also suggest that this unstirred boundary layer effect may contribute to fluid flow-induced regulation of ion channel

currents and should be considered while studying fluid flow-mechanosensitivity of ion channels. However, based on this hypothesis, it may be asked why some ion channel currents are not sensitive to fluid flow-dependent regulation if the unstirred boundary layer effect is an electrochemical rather than biological control. As briefly addressed above, this is probably because only ion currents through channels with large enough single-channel conductance and long enough open-time can be facilitated by fluid flow. That is, for the establishment of the unstirred layer in which the ion concentration is different from the average in bulk solution, flux in the membrane phase should be rapid enough compared to that in the aqueous phase[14]. We have recently suggested that the current through Kir2.1 channels, whose conductance and open time are sufficiently high, is facilitated by fluid flow *via* mechanisms of convective restoration of ion concentration in the unstirred boundary layer of cell membrane surface[11].

In conclusion, we present a method for measuring ion concentration in the unstirred boundary layer adjacent to the reference electrode and cell membrane surface with an open patch-clamp pipette. Besides emphasizing the importance of an agarose KCl bridge, this method also provides a way to account for the unstirred layer effect while interpreting fluid flow/shear force control of ion channels.

ACKNOWLEDGMENTS:

This research was supported by the Pioneer Research Center Program (2011-0027921), by Basic Science Research Programs (2015R1C1A1A02036887 and NRF-2016R1A2B4014795) through the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning, and by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI15C1540).

DISCLOSURES:

The authors have nothing to disclose.

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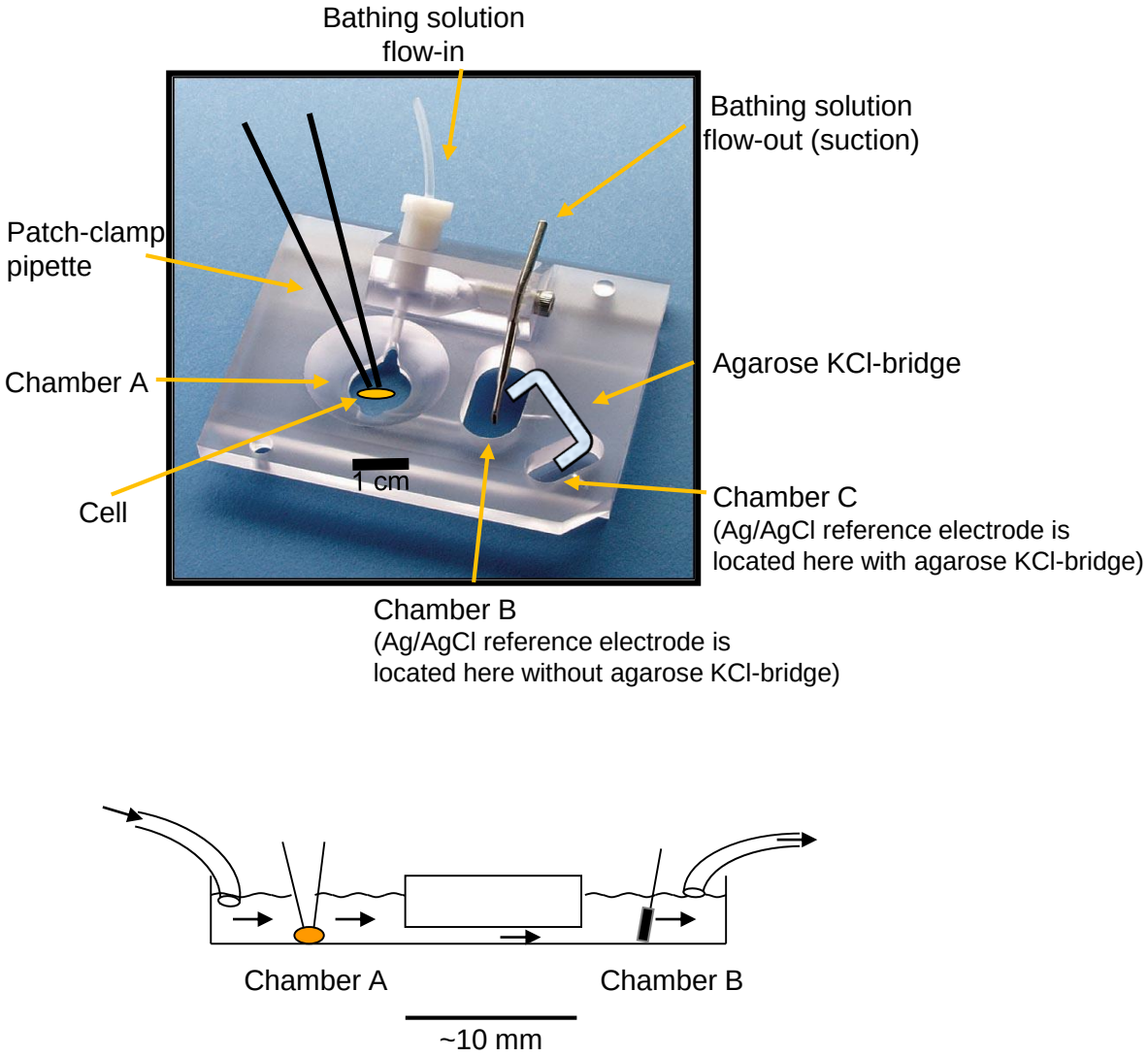


Figure 1.

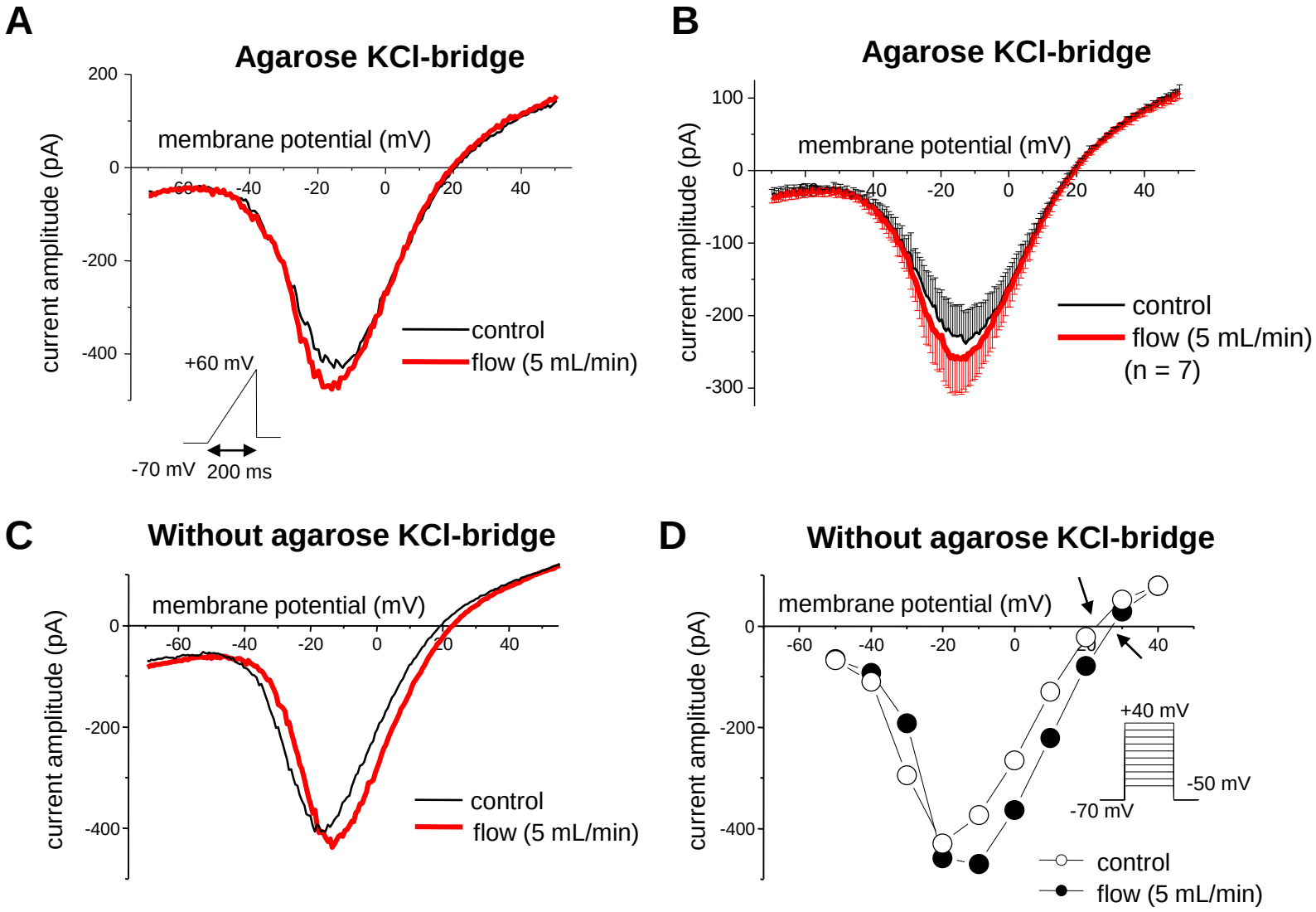
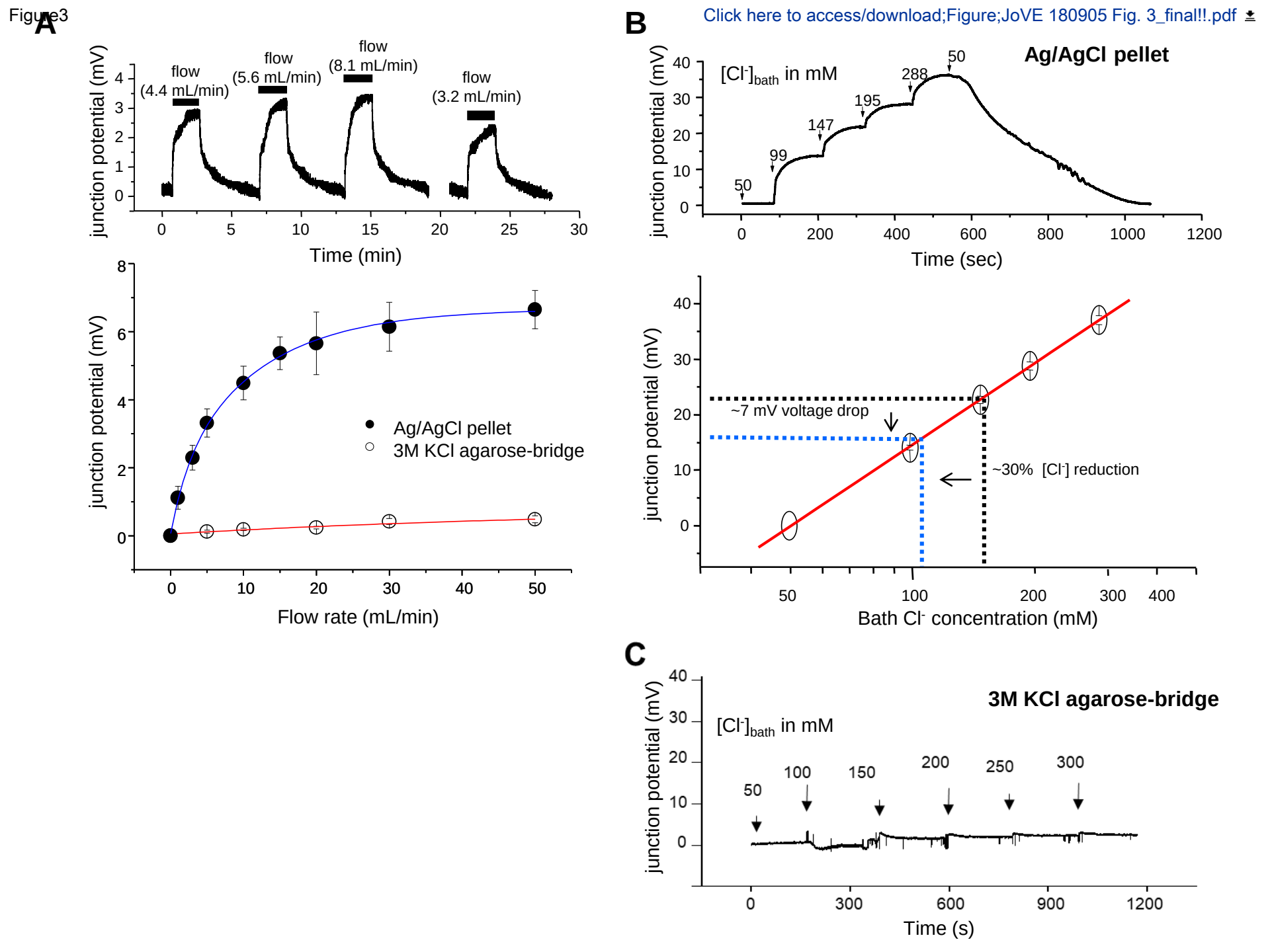


Figure 2.

Figure3



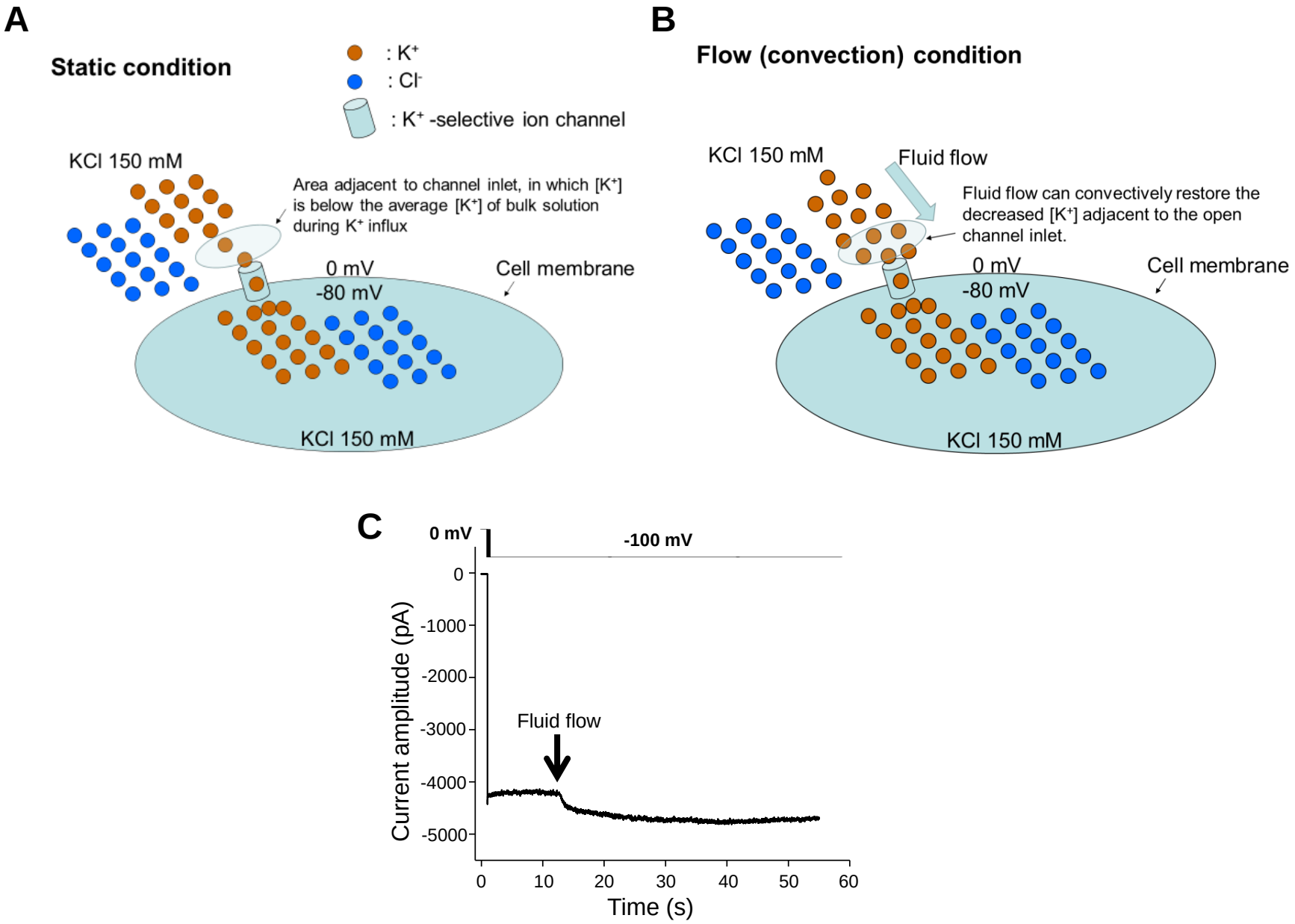


Figure 4.

Name of Material/ Equipment	Company	Catalog Number
RC-11 open bath chamber	Warner instruments, USA	W4 64-0307
Ag/AgCl electrode pellet	World Precision Instruments, USA	EP1
Agarose	Sigma-aldrich, USA	A9793
Voltage-clamp amplifier	HEKA, Germany	EPC8
Voltage-clamp amplifier	Molecular Devices, USA	Axopatch 200B
Liquid pump	KNF Flodos, Switzerland	FEM08

Comments/Description

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Author(s): Jae Gon Kim, Sang Woong Park, Kyung chul Shin, Bokyung Kim, Doyoung Byun, and Young Min Bae

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Dear editor and reviewers,

Thank you very much for reviewing our manuscript. We appreciate the helpful and constructive comments from the editor and the reviewers. We have responded to all comments raised by the editor and the reviewers, and revised the manuscript accordingly. The title of the manuscript has been changed to **"Measurement of ion concentration in the unstirred boundary layer with open patch-clamp pipette – implications in control of ion channels by fluid flow" from "Protocols for fluid shear force-regulation of ion channels in patch clamp recordings."** We hope that JOVE will find this revised manuscript acceptable.

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Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Answer) Thank you for the kind comment. We have proofread the manuscript which was edited by a professional English editor.

2. Please revise lines 92-99 and 192-197 to avoid previously published text.

Answer) Thank you. The lines have been revised.

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Answer) Thank you for the suggestion. Your instructions have been followed.

6. Figures: Please line up the panels better. Some panels are off-set in Figure. Please ensure that the panels are of the same dimensions if possible. Please also use consistent font size among panels in the same figure, if possible.
Answer) Thank you for the kind comment. We have edited the figure accordingly.

7. Figure 2: Please change “ml” to “mL” in figure.
Answer) Thank you. This has been corrected.

8. Figure 3: Please change “ml” to “mL”, and “sec” to “s” in figure.
Answer) Thank you. They have been corrected.

9. Please include a space between all numbers and their corresponding units: 1 M, 15 mL, 37 °C, 60 s; etc.
Answer) Thank you. They have been corrected.

10. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Warner Instruments, etc.
Answer) Thank you for the suggestion. This has been done.

11. 1.2.3: Please specify the temperature used.
Answer) Thank you for the comment. The temperatures have been specified.

12. 2.5: Please describe how the flow rate is measured.
Answer) Thank you. This has been described.

13. 5: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about analysis of outcome to the Results or Discussion section.
Answer) Thank you. This section has been moved.

14. Please discuss all figures in the Representative Results. However for figures showing the experimental set-up, please reference them in the Protocol.
Answer) Thank you for the suggestion. Your instructions were followed.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique

d) The significance with respect to existing methods
e) Any future applications of the technique
Answer) Thank you. We have edited the discussion to cover the items.

16. References: Please do not abbreviate journal titles.
Answer) Thank you. This has been corrected.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes a well know method to generate salt-bridges for grounding electrophysiology bath chambers. The authors report flow over the surface of the wire electrode results in an voltage offset. They extend this to suggest such an offset arises due to convective flow. They describe the use of a salt-bridge to remove the effect. The authors describe shear induced ion channel changes as due to an artifact. This artifact is from a calculation(Fig.5) of how convective flows alter the ionic concentrations at the proximity of ion channels. The authors propose that observed shear induced changes in ion channel conductance arise due to this artifact.

Major Concerns:
General Comments:
1.Convective flow effects on cellular boundary layer ionic concentrations are not an experimental artifact. Boundary layers are real phenomena of cellular solutions. If convective flow alters boundary layers, the effect should be more accurately described as a potential mechanism of shear alters ion channel behavior. If this hypothesis is true, then any ion channels subjected to flow and sensitive to ionic conditions would be expected to show responses to flow. However, multiple ion channels, sensitive to ionic concentrations, do not respond to shear stress. Examples of shear stress insensitive ion channels include ROMK (Shi et al., J. Biol. Chem. 291(27)p14012 (2016)); epithelial sodium channel mutations alter the responses to flow (Carattino et. al., J. Biol. Chem. 280 (6) p4393 (2005)); MEC-10 mutations alter shear responses (Shi et.al., J. Biol. Chem. 291(27) p14012 (2016); Kv1.1 (Hoger et.al., Proc. Natl. Acad. Sci. USA. 99(11) p7780 (2002)) and CFTR (Vitzthum et.al., Biochim. Biophy. Acta 1848 p 2942 (2015)). Additional shear stress mechanisms have been reported to include shear induced assembly of Kv1.5 (Boycott et al., Proc. Natl. Acad. Sci. USA. 110(41) E3955 (2013)) and intracellular calcium response of TrpM4 (Son et.al., J. Physiol. 594.11 p2985 (2016)). These examples of channels insensitive to shear are difficult to reconcile the proposed shear response hypothesis of convective flow altered ion layers.
Answer) Thank you for the critical and helpful comment. We understood the concern. In fact, we did not intend to suggest that the boundary layers adjacent to cell membranes are an experimental artifact. Our intention was to suggest that changes of ion concentration in the boundary layer should be considered in interpreting the control of ion current by fluid flow or shear force. However, we feel that the changes in the Cl^- concentration in the boundary layer adjacent to the reference electrode are a likely source of experimental errors or artifacts. In order to clarify our contention, we have extensively edited the manuscript,

including the title. In regard to the insensitivity of some ion channels to the fluid flow/shear force, a sufficiently fast flow rate through the ion channel pore is required to generate the unstirred flow rate. This point has been discussed in detail in the revised paper as follows:

The unstirred layer effect was originally suggested by Barry and his colleagues[12-15]. Here, we have further provided a way to estimate the real ion concentration in the unstirred layer by measuring changes in junction potential with open patch-clamp pipette. We also suggested that this unstirred boundary layer effect may contribute to fluid flow-induced regulation of ion channel currents and, hence, should be considered while studying fluid flow-mechanosensitivity of ion channels. However, based on this hypothesis, one might ask that if the unstirred boundary layer effect is an electrochemical control, rather than biological, why are some ion channel currents not sensitive to fluid flow-dependent regulation. As briefly addressed above, it is probably because only the ion currents through channels with big enough single-channel conductance and long enough open-time can be facilitated by fluid flow. That is, for the establishment of unstirred layer, in which the ion concentration is different from the average in bulk solution, flux in the membrane phase should be rapid enough compared to that in the aqueous phase[14]. We have recently suggested that the current through Kir2.1 channels, whose conductance and open time are high enough, is facilitated by fluid flow via a mechanism of convective restoration of ion concentration in the unstirred boundary layer of cell membrane surface[11].

2. Details are described below but modifying solutions with a naked silver chloride electrode will usually result in potential changes. This is not controlled for in the procedures. This could be the source of the voltage offset listed.

Answer) Thank you for the helpful comment. We agree that the changes in ion concentrations, (especially [Cl^-]) at the junction of the silver chloride electrode will result in the potential changes. As clarified in the revised manuscript, the purpose of the experiment shown in Fig. 3 was to estimate the actual concentrations of [Cl^-] in the boundary layer. As such, the graph shown in Fig. 3B is a '*stand curve*' for estimating the real [Cl^-] in the boundary layer of either reference electrode or cell membrane surface. From the graph in Fig. 3B and data on junction potential changes caused by fluid flow shown in Fig 3A, we may estimate the actual [Cl^-] in the unstirred boundary layer in the static condition. This concept has been better clarified in the revised manuscript.

Minor Concerns:

1. In Protocol 1, the use of a salt-bridge ground is well established and not a novel technique. Advantages of salt-bridge use are well known for avoiding solution-voltage issues. Changes in solution properties will result in voltage offsets.

Answer) We do agree with this comment. In the revised paper, we have clarified our hypothesis as well as the conclusions.

2. A missing control is a description of controlling chamber fluid depth. If fluid depth is altered by convective flow, then the fluid level on the recording pipette changes. This change can alter the electrical properties of the pipette.
Answer) Thank you for the comment. We did carefully maintain the fluid depth to ~2 mm.

3. The placement of the recording pipette must be down stream of the of the perfusion pipette to minimize turbulent flow over cell surface.
Answer) Thank you. The recording pipette was located downstream of the perfusion pipette. We modified Figure 1A in the revised paper to emphasize this point.

4. Finally, the direct application of flow from a pipette to a cell must have certain parameters met to insure laminar flow (Shi and Carattino, BioProtoc. 7(8) #2224 (2017)). Laminar flow is achieved by keeping the cell within one flow delivery pipette diameter distance from the pipette. The submerged pipe delivers laminar flow only within a short distance of the pipe mouth.
Answer) Thank you for the comment. We do agree that the flow may not be perfectly laminar. We used a commercially available patch-clamp chamber (RC-11 open bath chamber; catalogue # W4 64-0307) from Warner instruments company. Although the chamber may not have supplied ideal laminar flow, we believe that relatively stable laminar flow was applied. To demonstrate the geometry of the patch-clamp chamber more correctly, we have modified Figure 1 in the revised manuscript.

5. Description of flow directed on Ag/AgCl pellet should also describe preparation of pellet by chlorination. Only an adequate chlorinated pellet is a stable electrode.
Answer) Thank you. We agree with the comment. We purchased the Ag/AgCl pellet (catalogue #, EP1) from World Precision Instruments (WPI) company. We have further discussed this issue in the revised manuscript as follows:
In figure 3, we observed that the liquid-metal junction potential between Ag/AgCl reference electrode and bathing fluid was greatly dependent on the condition of the Ag/AgCl electrode. In fact, when the Ag/AgCl electrode was perfect in condition, changes in junction potential due to fluid flow was minimal (data not shown). However, poor chlorination of the Ag/AgCl electrode caused greater shift of the junction potential. Since the Ag/AgCl reference electrode is very susceptible to various external stimuli, such as ultraviolet light and oxidative stress, using an agar or agarose KCl-bridge is always recommended. Although changes in junction potential by fluid flow between bathing fluid and reference electrode is a potential source of experimental error, we could successfully estimate the real ion concentrations in the unstirred boundary layer by measuring the shift of junction potential under various fluid-flow rates (Figure 3A & B).

6. The results in Fig.4 are not an application of the methods described. These second messenger experiments (Fig.4 d) on a different cell line than reported in the literature for Kir2.1. These data are not necessary for communication of this method.

Answer) Yes, we agreed to this comment. Fig. 4d and the related description have been deleted in the revised manuscript.

7. In figure 3b, the experiment changes NaCl concentration. A missing control in these experiments is no correction for liquid-junction potential was described for when a change in NaCl is made. When changing ion concentrations compared to the pipette solution a liquid-junction potential will exist. Normally, these potentials are small. However, when large currents or large ionic solution changes occur (such as described in Fig 3) the potential can be 5-10mV ranges.

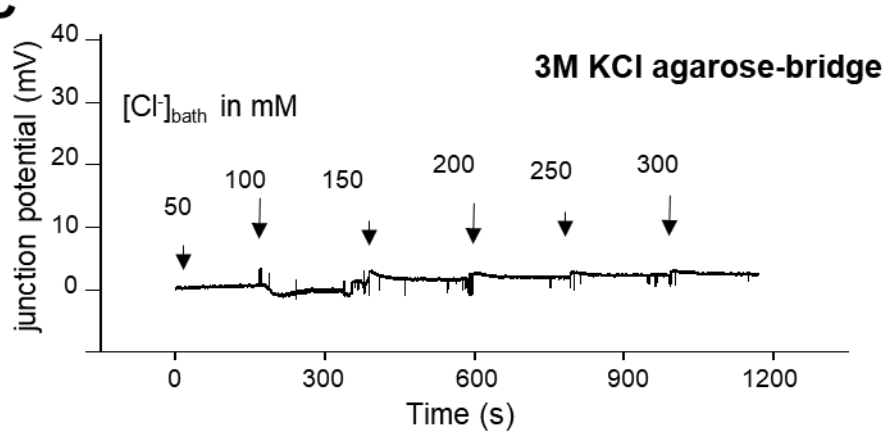
Answer) Thank you for the detailed comment. The liquid junction potential between various concentrations of NaCl bathing solution (ranging from 50 to 300 mM) and the 3M KCl-pipette solution has to be negligible. This point has been discussed in detail in the revised manuscript as follows:

The critical point in protocol 4, for preparing the standard curve for the estimation of real Cl^- concentration in the unstirred boundary layers from the shift of junction potential, is that the standard curve should be recorded under enough flow rate (30 mL/min, in this experimental setting). Although this flow rate is very fast in practical cases, faster the fluid, smaller is the concentration drop at the boundary layers (Fig. 3). In addition, the open pipette must be filled with high KCl, instead of regular pipette solution, for patch-clamp study to prevent the change in junction potential between the pipette and bathing solution.

Moreover, we added an experimetnal data, which shows minimum junction potetnail changes by the various concentrations of NaCl bathing soution as figure 3C with the following description:

Using a KCl agarose bridge, the junction potential was prevented from changing in a Cl^- concentration dependent manner (Fig. 3C), indicating that the junction potential change occurred between the bath solution and the reference electrode, not between the bath solution and pipette solution.

C


3M KCl agarose-bridge
[Cl-]bath in mM
50
100
150
200
250
300
junction potential (mV)
40
30
20
10
0
0
300
600
900
1200
Time (s)

Reviewer #2:

Manuscript Summary:

This manuscript discusses methods for preventing artifacts while studying flow-induced modulation of currents through ion channels. Flow-effect on ion channels has been an area of high-interest for over a decade. Therefore, describing the methodology that will minimize errors in data interpretation and analysis is important. The effect of fluid flow on liquid junction potential was shown by Park et al. in 2007. Park et al also showed that using an agar bridge eliminated the changes in liquid junction potentials with fuid flow. The current manuscript extends on the methodological details from the Park et al manuscript.

Answer) Thank you for the positive general evaluation.

Major Concerns:

The manuscript proposes that convective flow could be a source of variability in interpreting the data on flow-effect on ion channel function. There seems to be an issue with the model diagram used to explain this. The cartoon depicts that K flows in through Kir channels (5.3). At physiological potentials, K should flow from inside to the outside, thereby hyperpolarizing the cell membrane. Influx of K through Kir channels would need hyperpolarized potentials that are not physiological, and are less negative than K equilibrium potential.

Answer) Thank you for the helpful comment. Yes, the comment is correct. The example of inward current was used to ease understanding. The possibility of having an unstirred boundary layer with higher or lower concentrations of ions of interest depends on the direction of ionic current. We believe that the hypothesis stated in the manuscript is applicable both to inward and outward currents.

Minor Concerns:

I recommend that the authors convert at least some of the flow rate numbers to shear stress with the chamber parameters used in the study.

Answer) Thank you for the comment. We have edited the manuscript accordingly:

In **REPRESENTATIVE RESULTS:**

The voltage-independent facilitation of $VDCC_L$ current by fluid flow is a proper response of the $VDCC_L$ to the fluid or shear force. The 5 mL/min or approximately 0.004 m/s of fluid flow in the current experimental set up was estimated to represent approximately 0.1 dyn/cm^2 in terms of shear force (see discussion).

In **DISCUSSION:**

The shear force in the patch-clamp setting can be estimated from the following relationship[11]:

$\tau = (6\mu Q) / (bh^2)$ (2),

where, τ is the shear stress (N/cm^2), μ is the viscosity (0.001 N m/s^2 for water at 20°C), Q is the fluid flow rate (m^3/s), b is the chamber width (m), and h is the chamber height (m). When the fluid flow rate is 30 mL/min, the shear force in the patch-chamber shown in figure 1 is estimated to be ~0.75 dyn/cm^2 according to the above relation. This is quite a low shear force level compared with the physiological shear force; the endothelial cells in blood vessels can be subjected to shear forces of up to 40 dyn/cm^2 [1, 18, 19]. Therefore, provided that the ion channels are not sensitive to shear force < 0.75 dyn/cm^2, we can study the fluid flow/shear force sensitivity of ion channels after excluding the unstirred boundary layer effect by setting the control condition to be 0.75 dyn/cm^2. However, some ion channels, including Kir2.1, seem to be sensitive to shear force < 0.75 dyn/cm^2 [6].

"Fix the mode of the voltage-clamp amplifier to "I=0". Needs more explanation. How was this accomplished? What amplifier was used?

Answer) Thanks for the careful comment. We further explained on it as follows. Amplifier models are shown in the Material list excel file.

3.4. Fix the voltage-clamp amplifier to the current clamp mode ("*I=0*" or "CC").

Reviewer #3:

Manuscript Summary:

Review for 'Protocols for fluid shear force-regulation of ion channels in patch clamp recordings' by Jae Gon Kim et al.

The authors describe a clear and concise method, as to the importance of an agar salt bridge to the reference electrode when studying the role of shear stress on ion channel activity. This highlights an important method to reduce a recording artefact which can potentially influence a studies outcome. Below are a couple of minor comments which needs to be addressed before I can recommend this paper for publication.

Answer) Thank you for the positive general evaluation.

Major Concerns:

None

Minor Concerns:

1. In the 'static' conditions, how long was the solution static for? Do the authors have an idea on how fast the decrease in Cl- ion occurs around the reference electrode?

Answer) The time course of changes in junction potential (and thus the decrease of [Cl^-]) are detailed in Figure 3A. It is composed of fast and slow components. After the junction potential reaches a steady static condition, it may be maintained for a considerably long time (more than 30 min).

2. The authors could further explain the method to verify that the changes in voltage are liquid/metal junction potential, for those who are less experienced in electrophysiology set up. This however maybe clearer in the video recording

Answer) The revised manuscript has been largely edited including the title. We hope that the concerns expressed in the comment have been largely addressed in the revised manuscript.

3. Greater detail is needed in the representative results section, as to which cells are used to record the currents show.

Answer) Thank you for the helpful comment. The revised manuscript has been largely edited including the title. We believe that the concerns raised in the comment have been addressed in the revised manuscript.

4. The results in Figure 4 are not discussed or mentioned in the paper. A short rationale to why you are showing that data is all that is needed.

Answer) We thank you for the comment and apologize for the mistake. We have further clarified this issue in the revised manuscript and Figure 4d has been deleted.

5. The important topic raised in figure 5 needs more explanation to highlight the importance of flow when recording ion channel regulation. I.e. what could be seen as rundown could just be reduction in driving force.

Answer) Thank you for the critical comment. Based on this comment as well as comments of other reviewers, we have deleted Figure 5 in the revised manuscript. Instead, we added a discussion on the simulation study of ion concentrations in the unstirred boundary layer as follows:

Besides emphasizing the importance of using agar or agarose salt-bridge, another application of the method for estimating real ion concentration in the unstirred boundary layer is as follows: because plasmalemmal ion channels can function as ion-selective electrodes, just as the Ag/AgCl electrode functions like a Cl^- electrode, the real ion concentration in the unstirred boundary layer adjacent to the channel inlet at the cell membrane surface can be different from the average concentration of the bulk fluid. This difference in ion concentration between the bulk fluid and unstirred layer adjacent to cell membrane is the actual situation under clinical settings, and should be distinguished from the biological modulation of channel gating by fluid flow/shear force. Unfortunately, unlike the unstirred layer effect between the Ag/AgCl reference electrode and bathing fluid, we cannot fix the unstirred layer effect adjacent to the cell membrane surface in studying the regulation of ion channels by fluid flow/shear force. However, considering the observation that real ion concentration in the unstirred layer is approximately 70 % of that in bulk fluid (Fig. 3), we can make some amendments in the experimental data to distinguish the biological modulation of ion channels from the *electrochemical phenomenon of unstirred layer effect*. The real ion concentration in the unstirred layer at cell membrane surface was also expected to be approximately 70 % of the average concentration of the bulk bathing solution in our recent experimental and simulation study[10]. Since fluid flow restored the decreased ion concentration, it facilitated the Kir2.1 current, independent of cellular signaling [10]. In our previous study, current density was considerably high (2.5 A/m^2) with a high extracellular K^+ concentration and a high expression of Kir2.1 in RBL cells [10]. However, in the case of real cell membranes with various ion channel current density amplitudes, the unstirred layer effect at the cell membrane surface may depend greatly on the amplitude of ion channel current density. Besides, this may cause some ion channel currents, especially those with relatively lower current densities, to be insensitive to fluid-flow regulation, although the unstirred layer effect is regulated electrochemically and not biologically. This may affect the technique described in this study. Therefore, the possibility of developing a quantitative method that is adequate for correcting experimental results should be investigated in future studies.

Reviewer #4:

Manuscript Summary:

The authors describe the use of an agar/KCl bridge to minimize artifactual results arising from changing the flow rate of a superfusing solution. Changing the flow rate is used in the study of flow-mediated changes in ion channel activity. The authors provide both theoretical and experimental evidence supporting their ideas, as well as detailed protocols. By emphasizing the changes in electrode junction potentials that can arise at an Ag/AgCl bath electrode, the authors are making an important contribution towards best practice in electrophysiological experiments. The following issues require some attention.

Answer) Thank you for the positive general evaluation.

Major Concerns:

This manuscript is essentially about unstirred layers, also known as transport number effects, for which there is an important literature, particularly by PH Barry, none of which has been cited by the authors. Some of the earliest detailed studies were by Barry & Hope (1969) Biophys J 9:700-, & 729-, while Barry & Diamond (1984) Physiol Rev 64:763-872 is a major review that includes much original theoretical work by the authors for various experimental situations and therefore it is particularly relevant to the study of Kir channels discussed in this manuscript. Barry (1998) Biophys J 74:2903- provides additional clarification. Due to the much greater theoretical considerations and direct relevance to this manuscript, the authors should cite at least some of this literature.

Answer) Thank you very much for the critical comment. The authors found these references to be very helpful in further supporting our hypothesis. We have added the references to the revised manuscript. Moreover, inspired by the references, the manuscript, including the title, has been largely edited,

Page 1, Introduction, last paragraph: The authors should discuss what causes the depletion of Cl- from around the bath electrode. This is important since some of the membrane currents shown in this manuscript are up to many nA, and therefore much greater than what most experimenters would deal with. Does most of this depletion arise from the holding current? What happens if a cell is held at a positive potential and therefore an opposite holding current? If one records in current-clamp mode with no holding current (Protocol 3.4), why would the Cl- concentration against the bath electrode differ from that of the bulk solution?

Answer) Thank you for the critical comment. In fact, we are not certain whether we have a satisfactory answer for this query. We hypothesized the decrease in Cl^- concentration based on the deductions of previous experiments (Ref. 10 & 11) and this data shown in Figure 3 of the present manuscript.

In regard to the question raised in the comment, we tested the effect of fluid flow on the holding current and holding potential with negative, positive, and zero potentials. However, the shift was not dependent on the direction of the current. Rather, the shift was dependent on the condition (chlorination) of the Ag/AgCl electrode. When the reference Ag/AgCl electrode was in perfect condition, the shift of junction potential under I-clamp mode was minimal or negative (or slight outward shift of holding current under voltage-clamp mode). However, when the chlorination of the reference Ag/AgCl electrode was not perfect, the direction of shift in the holding currents was inward at both negative and positive holding potentials (please refer to accompanying additional data). Therefore, we edited the related description in the revised manuscript as follows.

One source of fluid flow-induced artifact in the patch-clamp recording is from the junction potential between the bath fluid and Ag/AgCl reference electrode[11]. It is generally believed that the liquid/metal junction potential between the bathing fluid and Ag/AgCl electrode is constant when Cl^- concentration of the bathing fluid is kept constant, considering the chemical response between the bathing solution and Ag/AgCl electrode to be:

$Ag + Cl^- \leftrightarrow AgCl + electron\ (e^-)$ (1)

However, in the situation where the overall electrochemical reaction between the bathing solution and Ag/AgCl reference electrode (equation 1) proceeds from left to right, the Cl^- concentration of the bathing fluid adjacent to the Ag/AgCl reference electrode (unstirred boundary layer [12-15]) may be much lower than that in the bulk of bathing solution, unless enough convectional transport is ensured. Using an old or non-ideal Ag/AgCl electrode with inadequate chlorination of Ag may increase such a risk. This fluid flow-related artifact at the reference electrode, in fact, can be simply excluded by placing a conventional agarose-salt bridge between the bathing fluid and reference electrode, since the artifact is based on the alteration in real Cl^- concentration adjacent to the Ag/AgCl electrode[11]. The protocol presented in this study shows how to prevent the flow-related junction potential change and measure the real ion concentrations in the unstirred boundary layer.

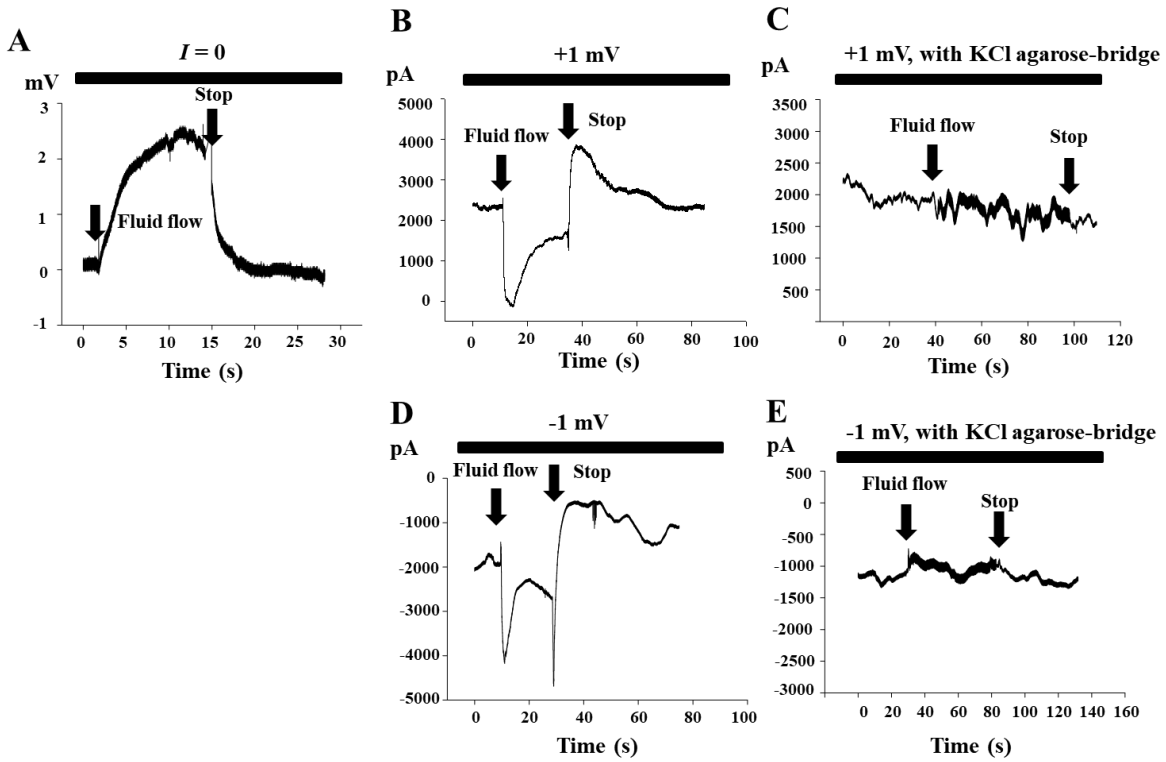


Figure. Representative tracings of the changes in the junction potential and junction current under current- and voltage-clamp, respectively. A, current clamp; B–E, voltage-clamp. A, B, and D, with Ag/AgCl electrode only; C, E, with KCl agarose-bridge.

It would be preferable if the order of the figures is closer to the order to which they are referred in the text.

Answer) Thank you. This has been done in the revised manuscript.

The authors refer to flow rates in ml/minute, as is done in most electrophysiological papers. However, the critical parameter for flow-mediated effects is the velocity of the solution (m/s), and this will depend on the geometry of the recording chamber. Therefore, in addition to the flow rate (ml/minute), it would be of use to the reader if the relevant velocities were also included (m/s).

Answer) Thank you. Suggested changes have been made to the revised manuscript.

Minor Concerns:

The list of materials at the end of the manuscript includes agarose. Therefore on page 2, Protocol 1.2.2, agarose should be included in addition to agar, along the lines of: "Weigh 10 g agar or agarose." Also, it would be useful if the % of agar or agarose in solution is given.

Answer) Thank you for the kind comment. In fact, we used agarose instead of agar, although either agar or agarose can be used for generation of the salt

bridge, it is conventionally called 'agar salt-bridge. However, for clarity and accuracy, we have corrected the agar to agarose in the revised manuscript.

Page 3, Protocol 2: Presumably an alternative way to stop and re-start the superfusion would be to use a pump and just it turn it off and on.
Answer) We agree with the comment. In fact, we also used the perfusion pump. We have added the description to the revised manuscript as follows:

2.8. Alternatively, to control the flow rate (for steps 2.3 – 2.6), use a perfusion pump. In that case, be careful to ensure a constant flow, rather than a pulsatile one.

Page 6, line number 270: Does "best fit by Nernst-equation" mean that the slope was such that the potential changed around 58 mV per ten-fold change in Cl- concentration?
Answer) Thank you for the comment. Basically, the answer is yes. However, the slope was not 58 mV, but 48.7 mV. We believe that, due to the selectivity of Cl^- over other ions, the junction potential generated between the Ag/AgCl and bathing fluid is not perfectly dependent on the Cl^- concentration. The slope value of 48.7 mV was from the best fit line. The 48.7 mV of slope indicates Cl^- dependency (or selectivity) of the Ag/AgCl reference electrode > 95% over other ion (in this case Na+) based on the GHK voltage equation. To make this point clearer, we added the following description in the revised manuscript:
The straight line in red represents the best fit by a modified Nernst-equation for equilibrium potential with a ten-fold slope of 49 mV. Owing to the finite selectivity of Na^+, compared to that of Cl^-, for generating the liquid/metal junction potential, the slope value of 49 mV, instead of 58 mV, produced the best fit in the junction potential-[Cl^-] relation at room temperature. The 49-mV slope indicates the Cl^- dependence (or selectivity) of the Ag/AgCl reference electrode > 95 % over the other ion (in this case, Na^+), according to the Goldman–Hodgkin–Katz voltage equation.

In Figure 3 and its legend, it may be less confusing and it would be more accurate if the total Cl- concentrations were used rather than just the NaCl concentrations.
Answer) Thank you for the careful comment, we have edited the manuscript accordingly.

In Figure 1, is there any advantage in having the accessory chamber for the reference electrode, compared with just inserting an Ag/AgCl wire directly into the agar bridge as is done by some researchers?
Answer) It is much more convenient to preserve the agar salt-bridge. In case the Ag/AgCl wire is directly inserted in the agar salt-bridge, we should be very careful not to create a space between the agar stat and the Ag/AgCl wire. But, in case a separate chamber is used for the bridge and the wire, the agar salt bridge can be just filled with the agar salt which is easy to maintain.

Page 5, line number 260: "pulses" implies a poorly defined time course. It would be better to use "steps".

Answer) Thank you. This has been corrected.

Figure 5A and its legend are a bit too cryptic. What is it trying to show? Why chose 5 mm?

Answer) The authors agree with this comment. Figure 5 has been deleted in the revised manuscript.

Figure 5 B requires some attention. What is it trying to show? What are the units "u" and "r"? The graph would be better if the data were plotted over a shorter x-axis range.

Answer) The authors agree with this comment, Figure 5 has been deleted. in the revised manuscript. This issue is discussed with the relevant citation (reference 10) as follows:

Besides emphasizing the importance of using agar or agarose salt-bridge, another application of the method for estimating real ion concentration in the unstirred boundary layer is as follows: because plasmalemmal ion channels can function as ion-selective electrodes, just as the Ag/AgCl electrode functions like a Cl^- electrode, the real ion concentration in the unstirred boundary layer adjacent to the channel inlet at the cell membrane surface can be different from the average concentration of the bulk fluid. This difference in ion concentration between the bulk fluid and unstirred layer adjacent to cell membrane is the actual situation under clinical settings, and should be distinguished from the biological modulation of channel gating by fluid flow/shear force. Unfortunately, unlike the unstirred layer effect between the Ag/AgCl reference electrode and bathing fluid, we cannot fix the unstirred layer effect adjacent to the cell membrane surface in studying the regulation of ion channels by fluid flow/shear force. However, considering the observation that real ion concentration in the unstirred layer is approximately 70 % of that in bulk fluid (Fig. 3), we can make some amendments in the experimental data to distinguish the biological modulation of ion channels from the *electrochemical phenomenon of unstirred layer effect*. The real ion concentration in the unstirred layer at cell membrane surface was also expected to be approximately 70 % of the average concentration of the bulk bathing solution in our recent experimental and simulation study[10]. Since fluid flow restored the decreased ion concentration, it facilitated the Kir2.1 current, independent of cellular signaling [10]. In our previous study, current density was considerably high (2.5 A/m^2) with a high extracellular K^+ concentration and a high expression of Kir2.1 in RBL cells [10]. However, in the case of real cell membranes with various ion channel current density amplitudes, the unstirred layer effect at the cell membrane surface may depend greatly on the amplitude of ion channel current density. Besides, this may cause some ion channel currents, especially those with relatively lower current densities, to be insensitive to fluid-flow regulation, although the unstirred layer effect is regulated electrochemically and not biologically. This may affect the technique described in this study. Therefore, the possibility of developing a quantitative method that is adequate for correcting experimental results should be investigated in future studies.

Page 2, Protocol 1.2.3: "Melt the agar" should be "Dissolve ....". Also, "on a hot plate.", not "in a hot plate."
Answer) Thank you for the comment. These terms have been corrected.

Page 3, Protocol 2.2: This sentence needs re-writing so it is easier to understand.
Answer) Thank you for the comment. The sentence has been re-written.

Page 3, Protocol 3.3: To clarify the situation, insert 'patch" and "electrode" so it reads "Place a patch pipette electrode ...".
Answer) Thank you for the comment. This has been corrected.

Page 3, Protocol 3.4: Include the fact that this is current-clamp mode (perhaps in brackets).
Answer) Thank you for the careful comment. This has been corrected

Page 5, line number 244: "Figure 5C & D" should be "Figure 4C & D".
Answer) Thank you for the kind comment. The related description was corrected and largely edited in the revised manuscript.

Page 5, line number 251: "subjected cell" should be "studied cell".
Answer) Thank you. This has been corrected.

Page 6, line number 284: "diameter = 12.5 μm" should be "radius = 12.5 μm", according to Figure 5.
Answer) Thank you. Figure 5 has been deleted in the revised paper.

Page 6, line number 302: It may be better to use the terms "chambers" instead of "rooms".
Answer) Thank you. The related term has been replaced in the revised manuscript as suggested.

Page 6, line number 304: Perhaps the authors mean "laminar flow" rather than "lamellar flow".
Answer) Thank you for the comment. The related term has been replaced in the revised manuscript.

Page 7, line number 321: "which is practically too fast" would be better phrased as "which, in practise, is too fast".
Answer) Thank you for the comment. The sentence has been edited.

Page 7, line number 331: "should" would be better as "would".

Answer) Thank you. Related sentence has been deleted.

In Figure 3A, it would be more consistent in terms of style, to have the tick marks as being horizontal rather than being angled.
Answer) Thank you for the comment. These have been corrected.

Figure 2B: Typo in "potential" in the label for the x-axis.
Answer) Thank you for the comment. This has been corrected.

In several places, "compared to" is used. Since similar items are being compared, "compared with" should be used.
Answer) Thank you. The manuscript has been edited by a professional English editor.

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