Further analysis of counterion permeation through anion-selective glycine receptor channels

Peter H. Barry,* Silas Sugiharto, Trevor M. Lewis and Andrew J. Moorhouse
Dept of Physiology; School of Medical Sciences; University of New South Wales; UNSW; Sydney, NSW Australia

The functional role of ion channels, which allow counterion permeation, depends critically on their relative anion-cation selectivity. From whole-cell patch clamp reversal potential measurements under dilution potential conditions, we have already shown that anion-cation permeabilities of anion-selective wild-type (WT) and mutant (with larger pore diameter) glycine receptor (GlyR) channels in the presence of Li⁺, Na⁺ and Cs⁺ counterions, were inversely correlated with the equivalent hydration diameter of the counterion, with chloride-cation permeability increasing as counterion equivalent hydration diameter increased with respect to the channel minimum pore diameter. Corrected for liquid junction potentials (LJPs; using ion activities), the previous chloride-cation permeabilities for the alkali cations were 23.4 (Li⁺), 10.9 (Na⁺) and 5.0 (Cs⁺) for the smaller WT channel. Further analysis to incorporate an initial offset potential correction, to fully allow for slight differences between internal cell composition and external control salt solution, changed the above permeability ratios to 30.6 (Li⁺), 11.8 (Na⁺) and 5.0 (Cs⁺), adding enhanced support for the inverse correlation between anion-to-counterion permeability ratio and equivalent hydrated counterion diameter relative to channel pore diameter (erroneously ignoring LJPs reduces each permeability ratio to about 4). Also, new direct measurements of LJPs (for LiCl and NaCl salt dilutions) using a 3 M KCl-agar reference salt bridge (with a freshly-cut end for each solution composition change) have shown excellent agreement with calculated LJPs (using ion activities), validating calculated LJP values. We continue to suggest that counterion cations permeate with chloride ions as neutral ion pairs.

**Introduction**

The functional role of ion channels, like those of the cys-loop ligand-gated ion channel superfamily, depends critically on whether they are predominantly permeable to cations or anions and on the magnitude of that relative selectivity. In many cases, the channels will allow the permeation of counterions, as is the case with oppositely charged Na⁺ permeating through the anion-selective GlyR channel. In order to determine how counterions permeate through such channels with a small minimum pore diameter, we recently investigated the relative Cl⁻ to counterion permeabilities for the alkali cations, Li⁺, Na⁺ and Cs⁺ for both the WT α1 GlyR channel (minimum pore diameter 5.3 Å) and the larger (P-2') mutant GlyR channel (minimum pore diameter 6.9 Å) (Sugiharto et al.;¹ henceforth referred to as ‘ref. 1’). Both channels had been expressed as homomeric GlyR channels in HEK293 cells and zero-current reversal potentials were obtained from whole-cell patch clamp measurements in different dilutions (from control 1.0 to ∼0.5 and ∼0.25 fractional dilutions of each salt, buffered with HEPES and kept isoosmotic with sucrose) of LiCl, NaCl and CsCl solutions (see Fig. 1 for the basic principle of the technique and Material and Methods, online Suppl. Material, or ref. 1, for further information). The shifts in reversal potential to 0.5 and 0.25 dilutions were corrected for...
liquid junction potentials (LJPs) calculated with the Windows version of the JPCalc program (Barry) for calculating liquid junction potentials, but using ion activities instead of ion concentrations, to correctly take into account the significant changes in ionic strength (see Suppl. Material). These calculations resulted in the relative anion-cation permeability ratio in LiCl solutions in ref. 1, with the same procedure being used for the other salts. The internal cell (pipette) solution composition was very similar, but not identical, to the external control 1.0 LiCl solution, which was sequentially replaced by a 0.5 LiCl solution, a 0.25 LiCl and a 1.0 LiCl solution. ‘Amp’ signifies a patch-clamp amplifier, ‘p’ the patch pipette, ‘c’ the cell with expressed GlyR channels and ‘sb’ a salt bridge reference with the same tip composition as the external control solution (1.0 LiCl). The full line in the inset I-V graph represents the I-V curve in either a 0.5 or 0.25 external diluted solution and -V rev, the zero current (reversal) potential for that solution. The dashed line represents the I-V curve in nominally symmetrical solutions (1.0 LiCl/LiCl). If the solutions had been symmetrical and there were no pipette tip potential, the reversal potential of this (1.0 LiCl/LiCl) curve should be zero. In practice, using the shift in reversal potential ∆V rev will eliminate any pipette tip potential, but will still leave a contribution from an initial offset potential due to slight differences in solution composition, which should be taken into account (see text). For other information see Material and Methods.

Figure 1. A schematic outline of the dilution potential methodology that was used to determine the relative anion-cation permeability ratio in LiCl solutions in ref. 1, with the same procedure being used for the other salts. The internal cell (pipette) solution composition was very similar, but not identical, to the external control 1.0 LiCl solution, which was sequentially replaced by a 0.5 LiCl solution, a 0.25 LiCl and a 1.0 LiCl solution. ‘Amp’ signifies a patch-clamp amplifier, ‘p’ the patch pipette, ‘c’ the cell with expressed GlyR channels and ‘sb’ a salt bridge reference with the same tip composition as the external control solution (1.0 LiCl). The full line in the inset I-V graph represents the I-V curve in either a 0.5 or 0.25 external diluted solution and -V rev, the zero current (reversal) potential for that solution. The dashed line represents the I-V curve in nominally symmetrical solutions (1.0 LiCl/LiCl). If the solutions had been symmetrical and there were no pipette tip potential, the reversal potential of this (1.0 LiCl/LiCl) curve should be zero. In practice, using the shift in reversal potential ∆V rev will eliminate any pipette tip potential, but will still leave a contribution from an initial offset potential due to slight differences in solution composition, which should be taken into account (see text). For other information see Material and Methods.

While both GlyR channels are expected to have a negatively charged selectivity filter region, it may be seen that as the equivalent hydrated diameter of the cation countercation increases, the value of P cl/P Na increases. This suggests that the countercation with the larger equivalent hydrated diameter has the smaller cation permeability. The effect is clearly much more dramatic for the smaller diameter WT GlyR channel, where at a minimum the Na+ would have to at least shed part of its hydration “shell” and the Li+ would have to lose much more of its hydration “shell”. In contrast, while the relative permeability sequence is the same for the larger P-2Δ GlyR channel, the magnitudes of the relative permeabilities are very much less.

Indeed, it might have been expected that the P cl/P Li value would have been much greater in the small diameter WT channel. Since the above countercion paper was published, it has become clear that under certain circumstances, the slight differences in composition between the almost symmetrical salt composition of the pipette and control bath solution would give rise to an initial offset potential. For the dilution potential measurements, in order to eliminate any small shifts in tip potential of the pipette, the shifts in reversal potential, V rev, in the two diluted (0.5 and 0.25 fractionally diluted) external bath solutions are measured relative to the control (1.0) bath solution (Fig. 1 inset). While using the shifts in reversal potentials automatically eliminates any pipette tip potential, it also eliminates any small offset potential across the cell membrane.

In addition, we validate the calculated values of the liquid junction potentials (LJPs; based on ion activities) with their experimental measurement using a new specially modified procedure, and also briefly outline the difference proper LJP correction makes to relative permeability data such as for the channels considered here.

Analysis

For the dilution and bi-ionic potential experiments, the reference salt bridge concentrations had the same ionic composition...
Kelvin, is discussed in Barry.6 In terms of analyzability and reliability of the zero current tions, respectively. The general applicability and reliability of the zero current GHK equation for relative permeabilities is discussed in Barry.4 In terms of analyzing our data it is reasonable to assume that the cell would be dialysed with the pipette solution and hence the internal activities of the cell are taken to be the same as those of the pipette solution. A problem arises in the control 1.0 LiCl (or NaCl or CsCl) solution, because in virtually symmetrical solutions the reversal potential is not zero. It has two possible components: a pipette tip potential and a small initial offset reversal potential, which we have termed an ‘offset potential’. The former is somewhat random, but the latter is well defined and determined by the slight difference in the composition of the cell and the external control solution. By measuring the shift in reversal potential during a salt dilution, the tip potential component would be eliminated, which is appropriate, but unfortunately so too would the small offset potential which is not appropriate. To correct for this and take into account the shift in reversal potential, ΔVrev, on going from the initial ‘symmetrical’ control solution to the test dilution solution, the following equation should be used:

$$\Delta V_{\text{rev}} = \Delta V_{\text{rev}} - \Delta V_{\text{Li}}$$

(1)

where ΔVrev represents the shift in liquid junction potentials calculated by the MS Windows version of JPCalc; which uses the generalized Henderson equation to calculate liquid junction potentials (Barry and Lynch) and in which ion activities are used to allow for the large changes in ionic strength in the different dilutions. The ΔV Li values are given in Table 2 for the salt dilutions.

For example, for dilution experiments in LiCl solutions, the zero current GHK equation expressed in activities and used to determine the reversal potential, Vrev, of glycine activated currents passing through homomeric α1 GlyR channels expressed in HEK 293 cells (recorded in a whole cell configuration) would be given by:

$$V_{\text{rev}} = \frac{RT}{F} \ln \left[ \frac{a_{\text{Li}}^0 + (P_{\text{Cl}}/P_{\text{Li}})a_{\text{Cl}}^0}{a_{\text{Li}}^i + (P_{\text{Cl}}/P_{\text{Li}})a_{\text{Cl}}^i} \right]$$

(2)

where Vrev is the reversal potential, R is the gas constant, T is the temperature in Kelvin, F is Faraday’s constant, and α is the activity of the ion, P is the permeability of the ion and superscripts ‘i’ and ‘o’ refer to the intracellular and extracellular solutions, respectively. The general applicability and reliability of the zero current GHK equation for relative permeabilities is discussed in Barry.4

Table 1. Ionic and equivalent hydrated diameters of Li+, Na+, Cs+ and Cl- ions and previous relative anion-cation permeabilities of these ions in two GlyR channels of different minimum pore diameter

| Cation counterions and chloride | Min. pore diameter | Li+ | Na+ | Cs+ | Cl- |
|--------------------------------|-------------------|-----|-----|-----|-----|
| Ionic diameter (Å)             |                   | 1.2 | 1.9 | 3.4 | 3.6 |
| Hydrated diameter (Å)          |                   | 7.5 | 6.5 | 5.0 | 5.0 |
| $P_{\text{Cl}}/P_{\text{t}}$  | (WT GlyR)         | 5.3 | 23.4 ± 2.8 | 10.9 ± 0.3 | 5.0 ± 0.5 |
| $P_{\text{Cl}}/P_{\text{Na}}$ | (P-2Δ GlyR)       | 6.9 | 6.0 ± 0.4 | 3.3 ± 0.2 | 1.9 ± 0.1 |

All data values and previous relative permeability values are from ref. 1.

Table 2. Relative permeabilities of the zero current GHK equation for relative permeabilities

| Cation counterions and chloride | Min. pore diameter | Li+ | Na+ | Cs+ | Cl- |
|--------------------------------|-------------------|-----|-----|-----|-----|
| Ionic diameter (Å)             |                   | 1.2 | 1.9 | 3.4 | 3.6 |
| Hydrated diameter (Å)          |                   | 7.5 | 6.5 | 5.0 | 5.0 |
| $P_{\text{Cl}}/P_{\text{t}}$  | (WT GlyR)         | 5.3 | 23.4 ± 2.8 | 10.9 ± 0.3 | 5.0 ± 0.5 |
| $P_{\text{Cl}}/P_{\text{Na}}$ | (P-2Δ GlyR)       | 6.9 | 6.0 ± 0.4 | 3.3 ± 0.2 | 1.9 ± 0.1 |

The dilution data from whole cell patch-clamp current-voltage curves for the WT and larger mutant (P-2Δ) GlyR channels expressed in HEK 293 cells, reported in ref. 1, were all initially re-analysed to determine the shifts in reversal potential in the absence of any LJP corrections and then the shifts in LJP values (Table 2) were applied to correct those reversal potentials as indicated in Tables 3 and 4 for both the smaller WT GlyR and the larger diameter P-2Δ GlyR channels.

It may readily be shown, that a failure to apply the LJP corrections results in a radical reduction in the channel selectivity especially in the WT channel.

Although the above approach, of first measuring the shifts in uncorrected dilution potentials and just applying the LJP corrections to those shifts, had a minor effect on accuracy, the major improvement to the analysis was to include the offset potential correction term (Eq. 3).

The resultant new relative permeability values for the above channels are shown in Table 5, where it may be readily seen, particularly for the WT GlyR channel with the smaller minimum pore diameter, that the offset potential correction has very markedly increased $P_{\text{Cl}}/P_{\text{Li}}$ from 24.3 to 30.6 and has moderately increased the value of $P_{\text{NO3}}/P_{\text{Na}}$ from 11.5 to 12.0, $P_{\text{Cl}}/P_{\text{Na}}$ from 11.1 to 11.8, though it has not noticeably affected $P_{\text{Cl}}/P_{\text{Na}}$. In comparison, for the P-2Δ mutant GlyR channel with the larger minimum pore diameter, where all the $P_{\text{Cl}}/P_{\text{cation}}$ values are much

as the initial bath solution (i.e., 1.0 NaCl), together with 3–4% agar. In each case, the shift in corrected reversal potential, ΔVrev, is related to the measured (raw) uncorrected value ΔV:}

$$\Delta V'_{\text{rev}} = \Delta V_{\text{rev}} - \Delta V'_{\text{Li}}$$

(1)
than the counterion affecting anion movement (Case 1), rather counterions were reflecting changes in relative permeability with different gating. In a biionic situation, NO₃ is minimally increased by Li⁺ and Cl⁻ components of conductance respectively, which since concentrations of cations and anions in both solutions are the same would be expected to be proportional to permeabilities (Suppl. Material). Ref. 1 showed that $G_{CsCl}/G_{LiCl} \approx 1.15 \pm 0.02$. With the new permeabilities incorporating offset potential corrections, it may be shown that $G_{CsCl}/G_{LiCl} = (1.209 \pm 0.02)/(1.037 \pm 0.005) = 1.17 \pm 0.03$ (from information with Tables 10 and 12 in Suppl. Material). Case 2, in which $g_{gCs} = g_{gLi} = g_{anion}$, predicts that:

$$G_{CsCl} = \frac{g_{gCs} + g_{Cl}}{g_{Li} + g_{Cl}}$$

$$G_{LiCl} = \frac{(g_{gCs}/g_{Cl}) + 1}{(g_{Li}/g_{Cl}) + 1}$$

$$= \frac{(P_{Cl}/P_{Cs}) + 1}{(P_{Li}/P_{Cl}) + 1}$$

(4)

Reference 1 showed that $G_{CsCl}/G_{LiCl} \approx 0.27 \pm 0.04$. With the new permeabilities incorporating offset potential corrections, it may be shown that $G_{CsCl}/G_{LiCl} = (6.03 \pm 0.49)/(31.6 \pm 4.4) = 0.19 \pm 0.05$ (from Tables 10 and 12 in Suppl. Material). Since, the experimental ratio of the whole cell slope conductances in CsCl to LiCl solutions, $G_{CsCl}/G_{LiCl}$ at this potential was measured to be 1.12 ± 0.02 (ref. 1), it is clear that the new permeability values are still virtually identical with the predictions of Case 1 (1.17 ± 0.03) and far from those of Case 2 (0.19 ± 0.04), to confirm that the anion component of conductance is independent of the counterion and that the counterion component is dependent on the cation.

In a particular experiment to compare $P_{anion}/P_{Na}$ for two anions with different relative permeabilities when measured in a bionic situation, NO₃⁻ was chosen for comparison with Cl⁻ (ref. 1), since it had been suggested by Franciolini and Nonner that if counterion movement were coupled to anion movement, then $P_{anion}/P_{Na}$ would

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Solutions & $\Delta V_{\text{rev}}$ (0.5) & $\Delta V_{\text{rev}}$ (0.25) \\
\hline
LiCl & -5.1 mV & -10.3 mV \\
NaCl & -3.1 mV & -6.4 mV \\
CsCl & 0.1 mV & 0.1 mV \\
NaNO₃ & -2.6 mV & -5.2 mV \\
\hline
\end{tabular}
\caption{Liquid junction potential corrections ($\Delta V_{\text{rev}}$) for dilution potential experiments, calculated using ion activities}
\end{table}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Salt (n) & $\Delta V_{\text{rev}}$ in mV & $\Delta V_{\text{rev}}$ (0.5) & $\Delta V_{\text{rev}}$ (0.25) \\
\hline
LiCl (6) & 7.8 ± 0.6 & 19.6 ± 0.4 & 12.9 ± 0.6 \\
NaCl (32) & 9.2 ± 0.2 & 19.1 ± 0.2 & 12.3 ± 0.2 \\
CsCl (6) & 8.1 ± 0.5 & 19.8 ± 0.2 & 12.3 ± 0.4 \\
NaNO₃ (7) & 9.7 ± 0.4 & 19.8 ± 0.2 & 12.3 ± 0.4 \\
\hline
\end{tabular}
\caption{Average WT GlyR dilution potentials in the absence and presence of LJP corrections}
\end{table}

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Salt (n) & $\Delta V_{\text{rev}}$ in mV & $\Delta V_{\text{rev}}$ (0.5) & $\Delta V_{\text{rev}}$ (0.25) \\
\hline
LiCl (7) & 5.0 ± 0.3 & 11.3 ± 0.7 & 10.1 ± 0.3 \\
NaCl (8) & 4.0 ± 0.4 & 7.0 ± 0.8 & 7.1 ± 0.4 \\
CsCl (7) & 3.3 ± 0.4 & 8.1 ± 1.0 & 8.0 ± 1.0 \\
\hline
\end{tabular}
\caption{Average P-2Δ GlyR dilution potentials in the absence and presence of LJP corrections}
\end{table}
Fig. 3 - 4

Equivalent biionic offset corrections were applied to the were applied? Equivalent biionic offset be true after offset potential corrections that previously shown that this appeared to be a constant for different anions. We had seen for dilution potential measurements of Suppl. Material). From Table 5 it is also seen for dilution potential measurements that after offset potential corrections the new \( P_{\text{NO3}}/P_{\text{Na}} \) (12.0 ± 0.5) was notionally even closer to \( P_{\text{Cl}}/P_{\text{Na}} \) (11.8 ± 0.4) than it had been in their absence.

It may be seen from Tables 2 – 4 that the LJP contributions to the average corrected \( \Delta V'_{\text{LJ}} \) (0.5) and \( \Delta V'_{\text{LJ}} \) (0.25) shifts could be very substantial. LJP calculations of dilution \( \Delta V'_{\text{LJ}} \)’s had previously been substantiated by measurements with Ag/AgCl reference electrodes previously for the three salts (Barry and Diamond)8 for 0.5 dilutions. We have since more directly measured \( \Delta V'_{\text{LJ}} \) (0.5) and \( \Delta V'_{\text{LJ}} \) (0.25) values for both the LiCl and NaCl dilutions using a special 3 M KCl reference salt bridge (Fig. 3, see details), in which the tip of the salt bridge was cut-off each time the solution composition was changed, necessary to eradicate history-dependent effects.8 The results are shown in Table 6.

Using this technique, the experimental and calculated \( \Delta V'_{\text{LJ}} \) values (using activities) were shown to agree within the 0.1 mV experimental error for both NaCl \( \Delta V'_{\text{LJ}} \) (0.5) and \( \Delta V'_{\text{LJ}} \) (0.25) and the LiCl \( \Delta V'_{\text{LJ}} \) (0.5) and within 0.2 mV for the much larger \( \Delta V'_{\text{LJ}} \) (0.25), thus validating the calculated values used in the analyses. The results also indicated that incorrectly using ion concentrations rather than activities overestimates the magnitudes of the LJP corrections, as would be predicted. In addition, we have calculated that erroneously ignoring LJP corrections completely results in the relative \( P_{\text{Cl}}/P_{\text{Na}} \) values decreasing to about 4 for each of the Li⁺, Na⁺ and Cs⁺ counterions.

This article has further analysed relative anion-cation permeability data from a previous whole-cell patch clamp study (ref. 1), designed to investigate the role of cation counterion permeation in two anion-selective GlyR channels expressed in HEK 293 cells. The major additional contribution of the analysis in this present article is the inclusion of an offset potential correction to allow for the small differences between the composition of the cell (pipette solution) and the control external bathing solution, in this case due to the extra presence of 5 mM EGTA, 2 mM CaCl₂ and additional LiOH (or

### Table 5. Re-analysed relative \( P_{\text{Cl}}/P_{\text{Na}} \) ratios for Li⁺, Na⁺ and Cs⁺ for both WT and mutant P-2Δ GlyR channels and \( P_{\text{Cl}}/P_{\text{Na}} \) for WT GlyR channels, with offset potential correction (New) compared to recalculated values without offset, all derived from the same original data in ref. 1.

| Channel          | Analysis       | \( P_{\text{Cl}}/P_{\text{Li}} \) | \( P_{\text{Cl}}/P_{\text{Na}} \) | \( P_{\text{Cl}}/P_{\text{Cs}} \) | \( P_{\text{Cl}}/P_{\text{NO3}} \) |
|------------------|----------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| WT GlyR          | New/Offset     | 30.6 ± 4.9 (6)                | 11.8 ± 0.4 (32)               | 5.0 ± 0.5 (6)                 | 12.0 ± 0.5 (7)                |
| WT GlyR          | No offset      | 24.3 ± 3.0 (6)                | 11.1 ± 0.3 (32)               | 5.0 ± 0.5 (6)                 | 11.5 ± 0.5 (7)                |
| P-2Δ GlyR        | New/Offset     | 6.3 ± 0.5 (7)                 | 3.3 ± 0.2 (8)                 | 1.9 ± 0.1 (7)                 | -                             |
| P-2Δ GlyR        | No offset      | 6.1 ± 0.5 (7)                 | 3.3 ± 0.2 (8)                 | 1.9 ± 0.1 (7)                 | -                             |

Because of additional improvements in the way the data was analysed, some of the values without offset correction, did increase permeability values above the old ref. 1 values (Cf. Table 1 and ref. 1). E.g., \( P_{\text{Cl}}/P_{\text{Na}} \) was increased by 0.9 in the WT channel and 0.1 in the mutant channel, and \( P_{\text{Cl}}/P_{\text{Na}} \) and \( P_{\text{Cl}}/P_{\text{Na}} \) values were each increased by 0.2 in the WT channel. As in the ref. 1 analyses, all the relative permeabilities were still averaged from values determined from individual dilution potential experiments, and are given as the mean ± SE, with the number of observations shown in parenthesis. It should be noted that the NaCl data set and new \( P_{\text{Cl}}/P_{\text{Na}} \) values for the WT GlyR only are the same as the control NaCl values in Sugiharto et al.6

**Discussion**

be a constant for different anions. We had previously shown that this appeared to be true (in the absence of offset potential corrections) for the WT GlyR.1 Would it still be true after offset potential corrections were applied? Equivalent bionic offset potential corrections were applied to the original bionic NaCl:NaNO₃ reversal potential data and the analysis indicated that \( P_{\text{NO3}}/P_{\text{Cs}} \) was unchanged from its old value of 1.6 ± 0.1 (see Eq. 11 and Table 15 of Suppl. Material). From Table 5 it is also seen for dilution potential measurements that after offset potential corrections the
NaOH or CaOH) in the pipette solution to buffer the internal pH in the presence of HEPES. The inclusion of this offset potential correction was to increase the anion-cation permeability ratio, the effect increasing as the anion-selective channel increased its selectivity. Thus, the increases in selectivity, upon inclusion of the offset potential correction, were greatest in the smaller diameter WT GlyR channel, with the largest change occurring for $P_{\text{Cl}^-}/P_{\text{Na}^+}$ which increased substantially from 24 to 31, followed by a fairly minimal increase for $P_{\text{Cl}^-}/P_{\text{Na}^+}$ and no appreciable change for $P_{\text{Cl}^-}/P_{\text{Na}^+}$. In comparison, for the larger P-2 GlyR channel, only $P_{\text{Cl}^-}/P_{\text{Na}^+}$ was noticeably changed and that only from 6.1 to 6.3. It should be noted that a similarly increased contribution of the initial offset potential correction was observed in the presence of external calcium, which if ignored further increased $P_{\text{Cl}^-}/P_{\text{Na}^+}$ for the WT GlyR channel. The updated relative counterion permeability values for the two channels can be compared with the sizes of the two GlyR channels and the ionic and hydrated sizes of the different ions in Figure 2, where the hydrated size of the Li$^+$ ion with its large equivalent hydrated size now has a much higher relative permeability in the smaller WT GlyR channel compared to Na$^+$ and Cs$^+$ (Fig. 6 of ref. 1).

We have also shown that the new relative permeability values continued to support the conclusions of the two other experiments reported in ref. 1. In one, the change in anion-cation permeability ratio between different cations was shown to be due to changes in the permeation of the counterion. In the other, a comparison of anion-cation permeability ratios for two

Table 6. A comparison of the experimental shifts in liquid junction potential (LJP) corrections ($\Delta V_L$), with respect to both the 1.0 LiCl and 1.0 NaCl solutions for dilution potential experiments for 0.5 and 0.25 dilutions with those calculated theoretically.

| Solution | Solution dilution | $\Delta V_L$ (Theor. - concs.) | $\Delta V_L$ (Theor. - activs.) | $\Delta V_L^m$ (Exp. corrected) (n) |
|----------|-----------------|-------------------------------|-------------------------------|----------------------------------|
| LiCl     | 0.50            | -5.4 mV                       | -5.1 mV                       | -5.1 ± 0.1 mV (12)               |
|          | 0.25            | -10.9 mV                      | -10.3 mV                      | -10.5 ± 0.1 mV (8)               |
| NaCl     | 0.50            | -3.4 mV                       | -3.1 mV                       | -3.0 ± 0.1 mV (6)                |
|          | 0.25            | -6.9 mV                       | -6.4 mV                       | -6.4 ± 0.1 mV (7)                |

The theoretical shifts in LJP ($\Delta V_L$) were calculated using concentrations (Col. 3) and activities (Col. 4) and compared with values measured “experimentally” ($\Delta V_L^m$; Col. 5) at 22°C. $n$ represents the number of bracketed measurements used for each pair of solutions (e.g., 1.0 LiCl/0.5 LiCl). It should be noted that the experimental and calculated $\Delta V_L^m$ values for NaCl dilutions in this table are the same as those given in the Supplementary Material of Sugiharto et al. 4

Figure 3. The experimental setup for measuring liquid junction potential (LJP) shifts in XCl salt solutions, where X represents the cation (XCl), for example, LiCl or NaCl. For XCl* test dilutions, a 150 mM XCl reference salt bridge is used for dilutions of test solutions from 1.0 XCl* solutions to 0.5 XCl* and 0.25 XCl* with the precise solution compositions as described in the Supplementary Material. The two Ag/AgCl electrodes contained either 150 mM XCl or 150 mM KCl, as indicated, with each electrode solution being gelled with 4% agar. Initially, at the start of an experiment, the 150 mM XCl salt bridge (gelled with 4% agar) was allowed to equilibrate for at least an hour with the appropriate 1.0 XCl* solution, which included 145 mM XCl, 10 mM HEPES buffered to pH 7.4 with XOH, glucose, sucrose, so that the tip of the 150 XCl electrode would be replaced by the 1.0 XCl* solution. The other part of the circuit included a 3 M KCl salt bridge gelled with 4% agar. As shown, the two electrodes were directly connected to the headstage input of a patch-clamp amplifier (Axopatch 200B). The tip of the 3 M KCl salt bridge was chopped off by at least 6 mm, whenever the composition of the test solution was to be changed to ensure that it had a fresh 3 M KCl composition at the end of the salt bridge in contact with the test solution (see Suppl. Material). As also indicated by the boxed equation, while the shift in LJP at this 3 M KCl salt bridge was dominated by the 3 M KCl, there was still an LJP correction at that salt bridge, indicated as $\Delta V_{3M}$, that needed to be applied to determine $\Delta V_L^m$ from $\Delta V_{exp}$. For the NaCl dilutions the $\Delta V_{act}$ values were 0.5 and 1.0 mV for the 0.5 and 0.25 NaCl dilutions, respectively, and for the equivalent LiCl dilutions, the $\Delta V_{act}$ values were 0.8 and 1.4 mV, respectively.

Table 6. A comparison of the experimental shifts in liquid junction potential (LJP) corrections ($\Delta V_L^m$), with respect to both the 1.0 LiCl and 1.0 NaCl solutions for dilution potential experiments for 0.5 and 0.25 dilutions with those calculated theoretically.
different anions still indicated that the counterion permeation was coupled to anion movement. With the increased $P_C/P_L$, value particularly, following the incorporation of the offset potential correction into the analysis, this article has provided enhanced support for the correlation between anion-to-counterion permeability ratio and the equivalent hydrated counterion diameter relative to channel pore diameter.

The presence of counterion permeation, particularly in small diameter ion channels also does raise the question of how cation counterions can pass through a negatively charged selectivity filter region (Fig. 2)? As already discussed our previous experiment with different anions that results in the same anion to cation permeability ratio and the equivalent hydrated counterion diameter relative to channel pore diameter. Therefore, we have also re-evaluated the ionic composition of the internal (pipette) solution and have come to the conclusion that since Ca²⁺ is bound by EGTA and virtually all is in the form of Ca-EGTA²⁻ at this pH (e.g., Dweck et al.), it is clear that EGTA²⁻ will be about 3 mM and Ca-EGTA²⁻ will be about 2 mM, so that from charge balance considerations that HEPES⁻ will be about 4 mM (see Table 1 in the Suppl. Material). The extracellular (bath) solutions used for the dilution potential experiments were the standard extracellular solution, resulting in approximately symmetrical XCl (referred to as: 1.0 NaCl, 1.0 LiCl or 1.0 CsCl); the solution where the concentration of XCl was reduced to half (0.5 XCl); and the solution where the concentration of XCl was reduced to about one quarter (0.25 XCl). E.g., the 1.0 LiCl (symmetrical) solution consisted of 145 mM LiCl, 10 mM HEPES and 10 mM glucose; the 0.5 LiCl solution consisted of 75 mM LiCl, 10 mM HEPES, 10 mM glucose, and 136 mM sucrose; and the 0.25 LiCl solution consisted of 37.5 mM LiCl, 10 mM HEPES, 10 mM glucose and 189 mM sucrose. All solutions were adjusted to pH 7.4 with measured amounts of XOH (approximately 4 mM in each external solution) and the concentration of ionic HEPES⁻ was estimated to be about 5 mM at pH 7.4. In order to determine the activities of the various solutions, we had to determine their ionic strength, $I$, to allow for the additional ions like HEPES⁻, EGTA²⁻ and Ca-EGTA²⁻. This was taken as:

$$I = \sum \frac{c_i z_i^2}{2}$$  \hspace{1cm} (6)

where $c_i$ is the concentration of each ion $i$ in the solution and $z_i$ is their valency. The estimates of ionic strength for all solutions are shown in Table 1 of the Supplementary Material.

Activity coefficients (γ) in each salt solution were determined by fitting a quadratic relationship between γ and $\sqrt{m}$ (where $m$ = molal salt concentration) using SigmaPlot 9 (Systat Software, Point Richmond, CA). For simplicity, $m$ was approximated by the molar (M) concentrations, the errors generally being less than 0.2% and the relative errors in final activities being much less than this. For NaCl, LiCl and NaN₃, the data were obtained from Robinson and Stokes (Suppl. Tables 8.7 and 8.9). For CaCl₂, the data were obtained from Table 2 in Cui et al. The following quadratic equations were used for the four salts:

$$\gamma = 0.9895 - 0.9659x + 1.0232x^2$$

$[\text{LiCl at } 20^\circ\text{C}]$  \hspace{1cm} (7)

$$\gamma = 0.9907 - 0.940x + 0.830x^2$$

$[\text{NaCl at } 20^\circ\text{C}]$  \hspace{1cm} (8)

$$\gamma = 0.9917 - 0.986x + 0.735x^2$$

$[\text{CsCl at } 25^\circ\text{C}]$  \hspace{1cm} (9)

$$\gamma = 0.9365 - 0.9202x + 0.5994x^2$$

$[\text{NaNO₃ at freezing point}]$  \hspace{1cm} (10)

where in each case $x = \sqrt{m}$ of the main salt (e.g., LiCl) and was approximated as $\sqrt{V}$. In order to calculate individual ion activities, the Guggenheim assumption can reasonably be assumed to apply (MacInnes; see also discussion in ref. 1), where for example in an LiCl solution, $\gamma_{Li} = \gamma_{Cl}$. It should be noted that the activity coefficient data at freezing point for NaNO₃ is within 0.4% of the value at 25°C for concentrations from 100 mM to 200 mM.

In each case the activity $a$ is related to the ion concentration, $C$, by:

$$a = \gamma C$$  \hspace{1cm} (11)

For practical purposes, there was only a very small proportion of divalent ions in the internal solutions and of HEPES⁻ in the external solutions, the same activity coefficient (that of the predominant salt, LiCl, NaCl, CsCl or NaNO₃) was used for all the ions in that solution at a particular ionic strength.

As already indicated, a further difference in approach in the new analyses for this article compared to ref. 1, was that rather than correcting the absolute value of each reversal potential for liquid junction
potentials before subtracting them, it was considered more accurate to use the raw (uncorrected) reversal potential values for each solution and then apply the calculated shift in liquid junction potentials to the shift in raw reversal potential values (as in Tables 3 and 4). This minimized the possibility of accumulating any small rounding errors in the calculations.

The data analyses for this present article were done using Sigma Plot 9 and Excel (Microsoft Corp.).

Further information about the data analyses and other experimental details are given in the online Supplementary Material.

The methodology for directly measuring liquid junction potentials is illustrated in Figure 3 and described in its legend.

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Note
Supplementary materials can be found at: www.landesbioscience.com/supplement/BarryCHAN4-3-Sup.pdf

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