Allosteric modulation of Ca\(^{2+}\) flux in a ligand-gated cation channel (P2X4) by actions on lateral portals

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Running title: Allosteric modulation of hP2X4R Ca\(^{2+}\) current

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Background: Ca\(^{2+}\) currents of ligand-gated ion channels are essential to cell signalling.

Results: We show that the calcium currents of P2X4 channels are subject to allosteric modulation.

Conclusion: The fixed negative charge of a single amino acid is required for the effects of ivermectin on permeability, flux, and current deactivation.

Significance: Allosteric modulators may provide therapeutic relief from symptoms of diseases such as peripheral neuropathy and hypertension.

SUMMARY

Human P2X receptors are a family of seven ATP-gated ion channels that transport Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) across cell surface membranes. The P2X4 receptor is unique amongst family members in its sensitivity to the macrocyclic lactone, ivermectin, which allosterically modulates both ion conduction and channel gating. In this paper, we show that removing the fixed negative charge of a single acidic amino acid (Glu\(^{51}\)) in the lateral entrance to the transmembrane pore markedly attenuates the effect of ivermectin on Ca\(^{2+}\) current and channel gating. Ca\(^{2+}\) entry through P2X4 receptors is known to trigger downstream signalling pathways in microglia. Our experiments show that the lateral portals could present a novel target for drugs in the treatment of microglia-associated disease including neuropathic pain.

The Ca\(^{2+}\) currents of ligand-gated ion channels (LGICs) play essential roles in cell signalling by regulating transmitter release, muscle contraction, and gene transcription (1,2). Most cells are exquisitely sensitive to [Ca\(^{2+}\)], and thus small changes in the amplitude of ligand-gated Ca\(^{2+}\) currents can lead to dramatic effects on the regulation of downstream Ca\(^{2+}\)-dependent signalling processes (3).

Recent works suggest that the ability to transport Ca\(^{2+}\) is not necessarily a fixed channel property of LGICs. This was first demonstrated in hippocampal neurons where PKA-dependent phosphorylation enhances NMDA-gated Ca\(^{2+}\) influx in dendritic spines by altering relative Ca\(^{2+}\) permeability, leading to facilitation of long-term potentiation (4). Other notable recent examples include PKC-dependent modulation of the Ca\(^{2+}\) permeability of polymodal TRPV1 receptors (5), and agonist-dependent modulation of the Ca\(^{2+}\) currents of TRPV1 (6) and TRPA1 receptors (7) receptors.

Despite these examples, a central unanswered question is whether the Ca\(^{2+}\) currents of LGICs are susceptible to allosteric tuning by drugs (8). If this
were so, then what is currently an interesting physiological phenomenon could be exploited pharmacologically. Thus, it might be possible to design drugs that allow channels to gate in response to their natural agonists, but that alter the Ca\(^{2+}\) flux of the conducted currents in a way that influences downstream cellular processes.

Here, we report that the macrocyclic lactone, ivermectin (IVM), reduces fractional Ca\(^{2+}\) current (\(Pf\%\)) and relative Ca\(^{2+}\) permeability (\(P_{Ca}/P_{Cs}\)) through native and recombinant P2X4 receptor-channels, thus demonstrating for the first time allosteric modulation of the Ca\(^{2+}\) current of a LGIC by an exogenously applied drug. This effect is absent in mutant human P2X4 receptors (hP2X4Rs) that lack the fixed negative charge of a specific acidic amino acid (Glu\(^{51}\)) in the lateral entrance to the transmembrane pore (9,10), and thereby identify a unique domain that might serve as a potential target for novel therapeutic agents.

**EXPERIMENTAL PROCEDURES**

**Cell culture of mouse cerebellar microglia** – Immortalized microglial C8-B4 cells were cultured in 35 mm dishes using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (American Type Culture Collection, Manassas, VA), 2 mM glutamine, and antibiotics as previously described (11). The night before an experiment, we added 1 \(\mu\)g/ml lipopolysaccharide (LPS) to the culture medium, and incubated the cells for an additional 12 hours. On the morning of the experiment, the cells were dispersed using a Trypsin/EDTA (1X) Hanks buffered saline solution (Sigma Co., St. Louis, MO), washed with DMEM, and replated onto polylysine-coated glass coverslips (Gold Seal; Becton-Dickenson) bathed in a solution of DMEM and LPS. Electrophysiological recordings began 2-6 hours later. We saw no ATP-gated currents in C8-B4 cells that were not exposed to LPS (11).

**Mutagenesis** – We studied recombinant hP2X4Rs that were made and expressed using conventional methods. Point mutations were engineered using the QuikChange Lightening Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and verified by automated DNA sequencing (Retrogen Inc., San Diego, CA). Plasmid cDNA was delivered to cultured HEK293 cells using Lipofectamine LTX (Invitrogen Corp., Carlsbad, CA).

**Data acquisition and drug application** – Whole-cell currents were recorded with low resistance (1-3 M\(\Omega\)), borosilicate glass pipettes (World Precision Instruments, Sarasota, FL) and AxoPatch 200B amplifiers (Molecular Devices, Sunnyvale, CA). Fura-2 fluorescence (510 nm excitation; 380 nm emission) was captured by a photomultiplier detection system (PTI, South Brunswick, NJ). Data were digitized at 10 kHz with Instrutech ITC-16 acquisition hardware (HEKA Instruments, Bellmore, NY) and AxoGraphX software (AxoGraph Scientific, Sydney, Australia). The stored data were analysed off-line using AxoGraphX and Igor Pro software (WaveMetrics, Lake Oswego, OR). Fast solutions changes were achieved using a SF-77B Perfusion Fast-Step system (Warner Instruments, Hamden, CT). The concentration of IVM was 10 \(\mu\)M except where noted otherwise.

**Patch clamp photometry** – The fraction of the total agonist-gated current carried by Ca\(^{2+}\) (i.e. \(Pf\%\)) was measured using the dye-overload method of Neher (12) and Dani (13). Our technique is described elsewhere in detail (14,15). In short, HEK293 cells transiently expressing P2X receptors were grown in 35 mm culture dishes, and then re-plated at low density onto poly-L-lysine coated glass coverslips (Gold Seal, Becton, Dickinson Co, Portsmouth, NH) 2-3 hr before the start of the experiment. Whole-cell current and fluorescence were recorded from adherent cells using a recording pipette containing (mM): 140 CsCl, 10 tetraethylammonium Cl, 3 CsOH, 10 HEPES, and 2 K 5-fura-2, at pH 7.3 (CsOH). The extracellular buffer contained (mM): 140 NaCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, and 10 HEPES, at pH 7.4 (NaOH). \(Pf\%\) was determined from the following equation:

\[
\text{Pf}\% = \frac{Q_{Ca}}{Q_T} \times 100
\]

\(Q_{Ca}\) (in nC) equalled \(\Delta F_{380}/F_{max}\), \(\Delta F_{380}\) is the change in fura-2 fluorescence caused by Ca\(^{2+}\) entry (measured in “bead units” or “BU”), and \(F_{max}\) is a proportionality constant determined in separate sets of experiments (equal to 23.68 BU/nC in most experiments). One BU equalled the average fluorescence of five Fluoresbrite 4.5 \(\mu\)M microspheres (Polysciences, Inc., Warrington, PA).
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PA). \(Q_T\) is the total integrated agonist-gated current recorded using patch clamp electrophysiology (in nC).

**Bi-ionic reversal potential measurements** – We used round cells that were detached from cultures dishes by mechanical dispersion to minimize space-clamp errors. Whole-cell membrane current was recorded using indifferent electrodes suspended in 3 M KCl agar bridges in contact with the bath solution, and the broken patch configuration of the whole-cell voltage-clamp technique. The solution in the recording pipette was (in mM): 150 CsCl, 10 EGTA, 10 HEPES, and pH 7.3 (CsOH). Relative Ca\textsuperscript{2+} permeability (\(P_{Ca}/P_{Cs}\)) was determined from the measured shift in reversal potential observed upon switching from the CsCl-based control solution to one composed of \(\text{mM}: 110 \text{ CaCl}_2, 10 \text{ glucose, 10 HEPES}, 2\text{ CaOH}_2\) (pH 7.4). We changed the membrane Cs\textsuperscript{+} (0.72 for 154 mM), and Ca\textsuperscript{2+} (0.26 for 112 mM), respectively. Relative Ca\textsuperscript{2+} permeability was (in mM): 150 CsCl, 10 EGTA, 10 HEPES, 2 CaOH\textsubscript{2} (pH 7.4). We changed the membrane voltage of cells bathed in each solution from -80 to 60 mV at a constant rate (1.4 V/s) before and during agonist application, and we measured the zero current level (\(E_{\text{Rev}}\)) from the leak-subtracted currents. Then, \(P_{Ca}/P_{Cs}\) was determined as:

\[
P_{Ca}/P_{Cs} = 4 \gamma_{Ca} \gamma_{Cs} / \gamma_{Ca} [Ca]_o \exp\left\{ \frac{\Delta E_{\text{Rev}}}{(RT/2F)} - 1 \right\}
\]

where \(\Delta E_{\text{rev}}\) equals \(E_{\text{rev,Ca}}\) minus \(E_{\text{rev,Cs}}\) (16). The \(\gamma_{Cs}\) and \(\gamma_{Ca}\) represent the activity coefficients for Cs\textsuperscript{+} (0.72 for 154 mM), and Ca\textsuperscript{2+} (0.26 for 112 mM), respectively.

**Homology Model** – We used the hP2X4R model built by Sébastien Dutertre (University of Queensland, St. Lucia, Australia) and based on the crystal structure of the zebrafish P2X4.1 receptor (zfP2X4.1R) (10), as previously described (9).

**Statistical Tests and Reports** – All data are presented as the mean ± S.E.M of at least six experiments. Drug treatments were analyzed by comparing data obtained before, during, and after drug treatment using the Student’s \(t\)-test function. Groups of data were analyzed by one-way ANOVA with significance determined from the Tukey’s protected multiple comparison test using Instat (Graphpad Software, Inc., La Jolla, CA). A \(p < 0.01\) was considered significant.

**RESULTS**

**IVM reduces the contribution of Ca\textsuperscript{2+} to the ATP-gated current of mouse cerebellar microglia.** The phenotypes of native homomeric P2X receptors are difficult to characterize because most tissues express more than one homologue and heteromeric receptors are common. We took advantage of the recent discovery of a pure population of mouse P2X4 receptors (mP2X4Rs) in LPS-stimulated C8-B4 microglia to study regulation of the Ca\textsuperscript{2+} current of a native ATP-gated ion channel (11). IVM is a positive allosteric modulator of rat (17) and human (18) P2X4 receptors. In recombinant hP2X4Rs, low concentrations (EC\textsubscript{50} \approx 0.3 μM) of IVM increase the maximum current evoked by saturating concentrations of ATP, and at higher concentrations (EC\textsubscript{50} \approx 2 μM), IVM also slows deactivation (18). Both of these effects are readily apparent in the native mP2X4R of activated C8-B4 cells (11). To determine the effect of IVM on \(Pf\%\), we applied ATP (30 μM, 2 s) while simultaneously measuring whole-cell current and fura-2 fluorescence. We found that the ATP-gated inward current (Fig 1A) was accompanied by a decrease in the fluorescence intensity emitted at 510 nm by fura-2 when excited by 380 nm light, indicative of rise in the intracellular [Ca\textsuperscript{2+}] (11). From these data, we calculated the \(Pf\%\) of the mP2X4R to be 15.4 ± 1.2%, (Table 1), as expected from previous results (11). Next, we bathed the cells in 10 μM IVM for 5 min, and then reapplied ATP. The ATP-gated current was larger in the presence of IVM, and deactivation was slower (Fig 1B). We again calculated the \(Pf\%\) from the change in fluorescence (Fig 1E), and found that the \(Pf\%\) was significantly smaller (7.0 ± 0.7%) than control (Table 1). The decrease in \(Pf\%\) was not due to dye saturation because the calibrated change in fluorescence (i.e., the \(Q_{Ca}\)) was a linear function of the total charge transfer across the surface membrane (i.e., the \(Q_T\) (Fig 1C,F)). Moreover, these linear functions show that the rise in free [Ca\textsuperscript{2+}] results solely from Ca\textsuperscript{2+} entry through the mP2X4R pore (19). To the best of our knowledge, this is the first report of allosteric modulation of the Ca\textsuperscript{2+} current of a native LGIC.

**IVM also decreases the Pf\% of recombinant hP2X4Rs.** Next, we used the wild-type recombinant hP2X4 to determine if the inhibition of \(Pf\%\) we saw in mouse cells was independent of tissue and conserved across species. Using recombinant receptors also allowed us to exploit
site-directed mutagenesis to study the molecular basis of the IVM effect. In keeping with published results (9,14,15), we found that the $P_f\%$ of the hP2X4R equalled 14.0 ± 0.7% in control conditions, and was not significantly different from that measured from mP2X4Rs (Table 1). We also found that IVM had predictable effects (18); the ATP-gated currents of the hP2X4R evoked in the presence of 10 µM IVM were larger than control and deactivated slower (Fig. 2A). More importantly, we recapitulated the effect of IVM on the $P_f\%$ of native mP2X4Rs in the recombinant hP2X4Rs. That is, the $P_f\%$ of the ATP-gated current of the hP2X4R fell to 8.1 ± 0.4% in the presence of IVM (Fig. 2B). IVM also reduced the $P_c/P_C$ of the hP2X4R, measured from the change in reversal potential of ATP-gated currents obtained in extracellular solutions containing predominately Na$^+$ or Ca$^{2+}$ (Fig. 2C). $P_c/P_C$ decreased from a control value of 4.3 ± 0.6 (n=8) to a new value of 2.1 ± 0.1 (n=3) in the presence of IVM.

Finally, we saw no effect of IVM on the $P_f\%$ of ATP-gated currents measured from HEK293 cells expressing either human P2X1 (hP2X1R) or rat P2X2 (rP2X2R) receptors (Fig. 2D; see also Table 1). These experiments support the claim that the effects of IVM are limited to P2X4Rs (20,21).

We attempted to rigorously determine the IC$_{50}$ of IVM on the $P_f\%$ and $P_c/P_C$ of the hP2X4R, but were hindered by the low aqueous solubility of the lactone in water at concentrations of >10 µM. Nevertheless, the effect of IVM was concentration-dependent because 3 µM IVM had a significantly smaller effect (11.1 ± 0.6%; n=9) on $P_f\%$ than that measured in the presence of 10 µM IVM (supplemental Fig. S1A). Lower concentrations of IVM (0.1, 1 µM) also appeared to have concentration-dependent effects (supplemental Fig. S1B), although the significance of these inhibitions were difficult to determine because of the limited range of attainable values of $P_f\%$ (~11-15%) measured in the presence of less than 3 µM IVM. Nevertheless, the data clearly show that a low (µM) affinity IVM binding site mediates the reduction in $P_f\%$ (18).

In the remaining experiments, we used site-directed mutagenesis of the recombinant wild-type (wt) hP2X4Rs and measurements of deactivation and $P_f\%$ to identify residues involved in the actions of IVM. We focused our study on sites previously shown to affect either channel gating (18,22,23) or $P_f\%$ (15,24).

Site-directed mutagenesis of TM1 has no effect on the ability of IVM to decrease $P_f\%$. P2X receptors have two transmembrane-spanning domains, designated TM1 and TM2. TM2 lines the pore (25-28), and regulates both the permeability and conductance of the ATP-gated current (14,29-32). In contrast, there is sparse data to support such a role for TM1 in cation permeability or conduction (24,33,34). One exception is the finding that the $P_f\%$ of the rP2X2R is significantly reduced when either of two sites in TM1 (Tyr$^{15}$ and Phe$^{44}$) is mutated to a hydrophobic alanine (24); similar results were recently reported for rP2X3 receptors (35). Mutagenesis of the homologous residues (Tyr$^{12}$ and Val$^{43}$; Fig 3A) of the hP2X4R to tryptophan reduces IVM sensitivity of deactivation (22,36), and so we hypothesized that these residues might be important for the effect of IVM on $P_f\%$. Therefore, we compared the three effects of IVM (potentiation of current, prolongation of deactivation, and reduction in $P_f\%$) on the ATP-gated currents of the wt receptor and the tryptophan mutants (Fig. 3B-D).

First, we found that tryptophan mutagenesis blunted the effect of IVM on the size of the hP2X4R, as expected from similar work using the rat P2X4 ortholog (36). The peak current amplitude of the wt hP2X4R measured in the presence of IVM (3 µM) was 4.2 ± 0.9-fold larger than control. In contrast, IVM caused a smaller potentiation of the hP2X4-Y42W current (1.2 ± 0.1 fold change) and inhibited the hP2X4-V43W current (0.6 ± 0.1) (Fig. 3B; Table 2). While these differences are significant, a firm conclusion cannot be drawn for the following reason. IVM increases the current amplitude of the wt hP2X4R primarily by increasing the open time/probability of the channel (18). If so, then two reasons could explain the smaller effects of IVM on the TM1 mutants. First, the mutations could limit the ability of IVM to increase open time. Second, the open time of the tryptophan-substituted mutants could be significantly greater than that of the wt receptor, which would limit the ability of IVM to further increase $P_o$. In-depth kinetic studies of the single channel currents of wt and mutant receptors are needed to identify the underlying cause, and
such studies are problematic because of the extensive rundown in channel activity that is an innate property of hP2X4Rs (9,18). Because the focus of the present study is the effect of IVM on Pf%, we did not pursue these single channel experiments here.

Next, we quantified deactivation by measuring the length of time it took for the agonist-gated current to fall from 90% of its peak current to 10% of its peak current following washout of ATP (called the t_{90-10%} time). We found that the fold-changes in the rates of deactivation of the mutant receptors were less pronounced in the presence of IVM (Fig. 3B), largely because tryptophan mutagenesis itself prolonged deactivation in the absence of IVM (Table 2). However, the absolute rates of deactivation of the wt and mutant receptors measured in the presence of IVM were not significantly different (see Table 2).

Finally, we measured the Pf% of these mutants before and after IVM (Fig. 3C,D). We found that replacing Tyr^42 or Val^43 of the hP2X4R with tryptophan had no significant effect on the Pf% in the absence of IVM, despite the fact that alanine mutagenesis at the homologous sites in the rP2X2R (Tyr^43 and Phe^44) significantly reduced Pf% (24). We also found that these mutations had no effect on the ability of IVM to attenuate the Pf% of the tryptophan-substituted receptors (Table 1). We draw two conclusions from these data. First, the fact that mutagenesis of Tyr^42 and Val^43 has no effect on Pf% supports the hypothesis that TM1 makes a smaller contribution to permeation than gating (24,35,37,38). Second, Tyr^42 and Val^43 are not involved in the reduction of the Pf% of hP2X4Rs by IVM.

The ability of IVM to decrease Pf% is attenuated by removing a fixed negative charge of the lateral portals. The Pf% values of P2X1 and P2X4 receptors are approximately twice that of all other family members (14), in part because two acidic amino acids (Glu^51 and Asp^331 of the hP2X4R) provide an electrostatic environment that interacts with Ca^{2+} (15). In our homology model of the hP2X4R, Glu^51 and Asp^331 lie just extracellular to the transmembrane domains and form part of the lateral portals that are the extracellular entrance to the pore (9) (Fig. 4A). IVM affects P2X4 currents by intercalating with the transmembrane domains (36), an interaction that affects the accessibility of engineered cysteines of the lateral portals to water-soluble thiol-reactive compounds (9). Thus, we hypothesised that IVM may reduce Pf% by changing the topology of the lateral portals in a way that lowers the capacity of Glu^51 and Asp^331 to facilitate Ca^{2+} flux.

To test this hypothesis, we investigated the IVM sensitivity of the hP2X4R mutants in which one or both of these acidic residues was replaced by the neutral amino acids that occupy the homologous positions (Gln^52, Ser^326) in the IVM-insensitive rP2X2R. In the first set of experiments, we measured the Pf% of the mutant hP2X4Rs in the absence of IVM. Consistent with published results (15), removing the single charge of either Glu^51 (E51Q) or Asp^331 (D331S) had no effect on Pf% (Table 1), whereas removing both charges (E51Q/D331S) significantly reduced Pf% to 7.6 ± 0.9%. After that, we measured the effect of IVM on the Pf% of the three mutant receptors, and found that the D331S mutant resembled the wild-type receptor because both showed equivalent reductions in Pf% in the presence of IVM (Fig. 4B,C). In contrast, IVM had no effect on the Pf% of either the E51Q (Fig. 4D) or the E51Q/D331S mutants (Fig. 4C), which shows that the ability of IVM to regulate Pf% is critically dependent on the presence of the fixed negative charge of Glu^51 in hP2X4R (Table 1).

Removing the fixed charge of Glu^51 also attenuates the effect of IVM on current deactivation. We then looked to see if removing the charge of Glu^51 and/or Asp^331 changed the ability of IVM to prolong deactivation following washout of ATP (Fig. 4E). We found that the t_{90-10%} of the E51Q/D331S mutant increased from a control value of 0.13 ± 0.01 s to new value of 1.7 ± 0.4 s in the presence of IVM. Although the prolongation is significant, it is ~10-fold shorter than the ~125-fold change observed for the wt receptor (Table 2). The effect of IVM on the t_{90-10%} of the ATP-gated current mediated by the E51Q mutant was also attenuated 10-fold, increasing from a control value of 0.32 ± 0.03 s in the absence of IVM to 3.9 ± 1.1 s in the presence of IVM. In contrast, IVM caused an ~80-fold increase in the time course of deactivation of currents mediated by the D331S mutant as the t_{90-10%} increased from 0.36 ± 0.05 s to 27.6 ± 6.4 s.

Removing the fixed charge of Glu^51 also reduced the effect of IVM on current amplitude (Table 2). Again, the reduced ability to potentiate
current could reflect either a change in the behavior of IVM or an innate property of
the mutant channels (see above); we did not further
investigate this effect.

Taken together, our data show that Glu\(^{51}\) is
necessary for the effect of IVM on \(Pf\%\) and
current deactivation. The strong correlation of the
effect of IVM on \(Pf\%\) and deactivation suggests
that a common final pathway may underlie both
effects.

**Insertion of Glu\(^{51}\) into zfP2X4.1R imparts
limited sensitivity to IVM.** The zfP2X4.1R lacks
the fixed negative charge of Glu\(^{51}\) and Asp\(^{331}\), and
has a \(Pf\%\) that is smaller than that of the hP2X4R
(14,15). Its sensitivity to IVM is unknown. We
mutated the zfP2X4.1R to place acidic amino
acids at sites equivalent to Glu\(^{51}\) and Asp\(^{331}\) of the
hP2X4R, and studied the effect of IVM on the \(wt\)
and the double-mutant (N34E/N334D) receptor.
Our experiments produced four noteworthy
results. First, in keeping with results obtained
using other P2X4 orthologs (21), we found that
IVM caused a \(~9\)-fold change in the peak current
amplitude of the \(wt\) zfP2X4.1R (Fig. 5A). To the
best of our knowledge, this is the first report of an
effect of IVM on the zebrafish ortholog. Second,
unlike its effects on the P2X4Rs of other species,
IVM had no significant effect on the time course
of current deactivation of the \(wt\) zfP2X4.1R (Fig.
5A; Table 2). However, IVM substantially
prolonged deactivation of the N54E/N334D
double charge mutant (Fig. 5B), as the \(t_{90-10}\%\)
changed from a control value of 0.4 ± 0.1 s in
control conditions to 14 ± 2.5 s in the presence
of 3 \(µM\) IVM. Again, in concordance with our results
with the hP2X4R (see Fig. 4E; these data suggest
that the effect of IVM on current deactivation
requires the presence of fixed charge in the lateral
portal of P2X receptors. Third, inserting the fixed
negative charges of glutamate and aspartate into
the zfP2X4.1R had no effect on \(Pf\%\) measured in
the absence of IVM (Fig. 5C). This outcome was
unexpected because the \(Pf\%\) of the rP2X2R, which
also lacks the two essential acidic amino acids,
doubles following insertion of glutamate and
aspartate at the equivalent sites (Fig. 5C; (15)).
Fourth, IVM had no effect on the \(Pf\%\) of currents
evoked in cells expressing either the \(wt\) zfP2X4.1R
or the zfP2X4.1-N54E/N334D double mutant
(Fig. 5C). Why insertion of fixed charge failed to
increase \(Pf\%\) or impart IVM sensitivity to the \(Pf\%
of the zfP2X4.1R is presently unknown.

**A double charge mutant of rP2X2R is IVM
insensitive.** In a final set of experiments, we
looked to see if we could impart full or limited
sensitivity to IVM by inserting “Glu\(^{51}\)” into an
IVM-insensitive, non-P2X4 receptor. The hP2X1R
has acidic charge at the homologous site and a
relatively high \(Pf\%\) but is insensitive to IVM (see
Fig. 2D), which suggests that Glu\(^{51}\) is not the sole
determinant of IVM sensitivity. To test this
hypothesis, we measured the IVM sensitivity of a
double rP2X2R mutant (Q52E/S326D) that
contains fixed charge at sites equivalent to Glu\(^{51}\)
and Asp\(^{331}\). We found that current amplitude and
the rate of deactivation of the double mutant
rP2X2R-Q52E/S326D were not affected by IVM
(Fig. 5D). The \(Pf\%\) of rP2X2R-Q52E/S326D was
similarly unaffected (Fig. 5C).

The following picture therefore emerges when
all of the data are considered together. We
conclude that the fixed negative charge of Glu\(^{51}\)
is required for the slowed deactivation and reduced
\(Pf\%\) caused by the action of IVM on hP2X4Rs.
However, Glu\(^{51}\) does not bestow IVM sensitivity
by itself, as the effects of IVM sensitivity are
restricted to P2X4Rs. The later finding suggests
that additional structural loci unique to P2X4Rs
are required for the multiple effects of IVM.

**DISCUSSION**

IVM has two well-described effects on P2X4R
current. First, it potentiates whole cell current by
binding to a high affinity (nM) site, and second, it
prolongs deactivation by binding a low affinity
(\(µM\)) site (17,18,39). In the present report, we
show for the first time that IVM also reduces the
contribution of Ca\(^{2+}\) to the ATP-gated current of
native and recombinant P2X4Rs. Further, the
effects of IVM on deactivation and \(Pf\%\) are
markedly attenuated by removing the fixed
negative charge of a single acidic amino acid
(Glu\(^{51}\)) in the extracellular entrance to the
transmembrane pore. Our finding that drugs
modulate the ATP-gated Ca\(^{2+}\) current through
actions on the lateral portals provide further
support for the idea that these domains are flexible
and move as the channel opens (9,18). Drugs that
alter these movements may therefore provide
therapeutic relief from symptoms of diseases such
as peripheral neuropathy (40) and hypertension
(41).
Rather than point to a third and distinct action of IVM on P2X4R current, our data suggest that a common transduction pathway may underlie the effects of IVM on Ca\(^{2+}\) flux and current deactivation, for the following two reasons. First, although the limited solubility of IVM in water prevented construction of a complete concentration-response curve, the available data suggest that IVM binds to a low affinity (µM) site to mediate its effect on Pf% (see supplemental Fig. S1). The micromolar potency of IVM at this site is similar to the estimated EC\(_{50}\) for the low affinity effect of IVM on deactivation. Second, removing the fixed negative charge of Glu\(^{51}\) substantially attenuates the effects of IVM on both Pf% and current deactivation, thus demonstrating a common structural locus in the lateral portals for both effects. Again, the lateral portals are flexible and move when the channel opens (9,42). Our new data suggest that this movement may play a role in regulating the nature of cationic flux through the channel.

We speculate that the mechanism by which IVM attenuates Pf% and deactivation in P2X4Rs involves pushing the channel into a non-native conductance state with a lower \(P_{Ca}/P_{Na}\). This hypothesis is partially supported by the single channel data of Priel and Silberberg (18) who showed that micromolar concentrations of IVM increase mean open time, open probability, and single channel conductance of the hP2X4R channel. The increase in conductance suggests that IVM decreases \(P_{Ca}/P_{Na}\) and Pf% by increasing Na\(^+\) permeability and flux. While this may be so, it cannot fully explain the magnitude of the effects described here. That is, a 100% increase in the size of the Na\(^+\) current is required to fully explain the observed 50% reduction in Pf% by IVM, which is far greater than the ~20% increase in single channel conductance reported by Priel and Silberberg (18). A concomitant decrease in Ca\(^{2+}\) permeability and flux is therefore required too.

Our data strongly suggest that Glu\(^{51}\) lies in a transduction pathway that mediates the low affinity effects of IVM. However, the data also imply that additional domains are involved in the selective effects of IVM on P2X4Rs. For example, the double-mutant P2X2R (Q52E/S326D) has a glutamate at a position analogous to the Glu\(^{51}\) of P2X4R, but neither the Pf% nor the deactivation was altered by IVM. Similarly, wt hP2X1Rs have the equivalent charges, but are unaffected by IVM. In contrast, the ability to prolong deactivation by IVM was successfully conferred to the zfP2X4R by introducing fixed charge at the position analogous to Glu\(^{51}\) of the hP2X4R. Interestingly, our data suggests that the wild type zfP2X4R is still sensitive to the high affinity effects of IVM on current amplitude, even though its deactivation lacks sensitivity to IVM. Further experiments comparing the zebrafish and mammalian P2X4R channels may help to tease apart the structural loci underpinning the separate effects of IVM on channel function, and explain why the effects are specific to P2X4R-like receptors.

Finally, we previously showed that the amplitude of the Ca\(^{2+}\) current of rat and human P2X1 and P2X4 receptors is highly dependent on the two relevant fixed charges of the lateral portals discussed here (15). The zfP2X4.1R lacks these acidic residues, and has a lower Pf% than its mammalian counterparts. However, unlike our published results with rP2X2Rs (15), we did not record an increase in Pf% when carboxylates were added to the relevant positions in the zfP2X4.1R. We cannot fully explain the discrepancy between the rP2X2R and zfP2X4.1R results. Perhaps other, undiscovered domains contribute to ion selection, and these domains are missing in the zfP2X4.1R. In addition, due to the low current densities achieved with maximal stimulation of these receptors in HEK-293 cells (in the absence of IVM), we used long exposures to ATP (5-10 s) to a Ca\(^{2+}\) flux that was large enough to allow accurate measures of Pf%. These long applications may push the receptor into the I\(_2\) permeability state (43), which may have different Pf% properties.

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|                | control (%) | IVM (%) |
|----------------|-------------|---------|
| mP2X4R (C8-B4 cells) | 15.4 ± 1.2 (5) | 7.0 ± 0.7 (6)* |
| rP2X1R         | 11.7 ± 0.6 (3) | 10.0 ± 0.9 (3) |
| rP2X2R         | 7.3 ± 0.3 (10) | 7.4 ± 0.6 (4) |
| hP2X4R         | 14.0 ± 0.7 (22) | 8.1 ± 0.4 (14)* |
| hP2X4R-Y42W    | 17.5 ± 1.7 (7)  | 11.5 ± 1.8 (5)* |
| hP2X4R-V43W    | 13.9 ± 0.7 (5)  | 9.7 ± 0.9 (6)* |
| hP2X4R-E51Q    | 11.8 ± 0.3 (7)  | 9.9 ± 0.4 (6) |
| hP2X4R-D331N   | 11.3 ± 0.9 (5)  | 4.3 ± 0.7 (5) |
| hP2X4R-E51Q/D331N | 7.6 ± 0.9 (8)  | 7.0 ± 1.0 (6) |
| zfP2X4.1R      | 7.2 ± 1.3 (5)  | 6.7 ± 1.1 (7) |
| zfP2X4.1R-N54E/N334D | 6.3 ± 0.8 (8)  | 5.3 ± 0.5 (11) |

Table 1 – Effect of IVM on Pf%.
Asterisks denote a significant difference between control and IVM. The number of trials for each experiment is indicated in parentheses.
| Protein       | Fold-change in peak current by IVM | Deactivation rate control (s) | Deactivation rate IVM (s) | Fold-change in deactivation |
|--------------|-----------------------------------|-----------------------------|---------------------------|---------------------------|
| hP2X4R       | 4.2 ± 0.9 (15)                    | 0.14 ± 0.01 (18)            | 17.4 ± 2.4 (14)           | 124.3                     |
| hP2X4R-Y42W  | 1.2 ± 0.1 (10)                    | 7.9 ± 1.1 (17)              | 14.2 ± 2.7 (9)            | 1.8                      |
| hP2X4R-V43W  | 0.6 ± 0.1 (8)                     | 3.0 ± 0.4 (9)               | 19 ± 3.3 (5)              | 6.3                      |
| hP2X4R-E51Q  | 2.4 ± 0.5 (8)                     | 0.32 ± 0.03 (11)            | 3.9 ± 1.1 (7)             | 12.2                     |
| hP2X4R-D331S | 6.9 ± 1.5 (5)                     | 0.36 ± 0.05 (13)            | 27.6 ± 6.4 (5)            | 76.7                     |
| hP2X4R-E51Q/D331S | 2.2 ± 0.4 (6)        | 0.13 ± 0.01 (11)            | 1.7 ± 0.4 (5)             | 13.1                     |
| zfP2X4R      | 8.7 ± 3.5 (5)                     | 0.13 ± 0.05 (4)             | 0.6 ± 0.1 (4)             | 4.6                      |
| zfP2X4R-N54E/N334D | 40.1 ± 11.0 (4)   | 0.4 ± 0.1 (4)               | 14.0 ± 2.5 (4)            | 35                       |

Table 2 – Effect of IVM on peak current and deactivation

Deactivation rates are the \( t_{90-10\%} \) times measured as explained in the text. The number of trials for each experiment is indicated in parentheses.
Figure 1. IVM reduces the Pf% of C8-B4 microglia. ATP-gated whole-cell current was recorded from mP2X4Rs of C8-B4 cells using patch pipettes containing the Ca\(^{2+}\)-sensitive dye, fura-2. ATP (100 µM) evoked an inward current (A) and a change in F\(_{380}\) (B, blue trace). QT (B, black trace) was determined by integrating the ATP-gated whole-cell current. The contribution of Q\(_{\text{Ca}}\) to QT is marked by the grey shaded area of Panel B. Panel C shows that Q\(_{\text{Ca}}\) is a linear function of QT. Panels D, E and F show similar data obtained after a 5 min application of IVM (10 µM). IVM increased peak current amplitude and prolonged deactivation (D), and at the same time decreased Pf% (E). The decrease in Pf% was not caused by saturation of fura-2 because Q\(_{\text{Ca}}\) remained a linear function of QT (F).
**Figure 2. IVM reduces the $Pf\%$ of recombinant hP2X4Rs.** Whole-cell currents were recorded from HEK-293 cells using electrodes containing fura-2. ATP (30 µM) evoked inward currents (A) and changes in fura-2 fluorescence (B) in the absence (left traces) and presence (right traces) of IVM (10 µM). Panel C shows the effect of IVM on $P_{Ca}/P_{Na}$. Whole-cell current was recorded during voltage ramps to HEK293 cells expressing hP2X4Rs in the absence (left panel) and presence (right panel) of 10 µM IVM. Blue traces were obtained in high Na$^+$ extracellular solutions. Red traces were obtained in high Ca$^{2+}$ extracellular solutions. The solid black lines are the polynomial fits to the raw data. Panel D shows the pooled data for the effect of IVM on the $Pf\%$ of P2XRs. IVM decreased the $Pf\%$ of native and recombinant P2X4Rs but had no effect on hP2X1Rs or rP2X2Rs.
Figure 3. Site-directed mutagenesis of TM1. Panel A shows the location of Tyr\textsuperscript{42} (red spheres) and Val\textsuperscript{43} (blue spheres) along TM1 (green) in a homology model of the hP2X4R. The left cartoon shows the channel viewed parallel to the membrane. The inner and outer limits of the membrane are marked with lines. The right cartoon shows the protein viewed parallel to the three-fold axis of symmetry from the intracellular side of the membrane. TM1 (green) lies lateral to TM2 (brown) and the pore. Panel B shows the effect of IVM (10 \( \mu \)M) on the ATP-gated (3 \( \mu \)M) currents of the wt and mutant receptors. Panel C and D show ATP-gated (30 \( \mu \)M) current and fluorescence traces recorded in the presence and absence of IVM. These mutations had no significant effect on Pf\% by comparison to the wt hP2X4R, and no significant effect on the ability of IVM to reduce the Pf\%. 
Figure 4. Site-directed mutagenesis of the lateral portals. Panel A shows the location of Glu51 (red spheres) and Asp331 (blue spheres) in our homology model of the hP2X4R. The left cartoon shows the receptor viewed parallel to the membrane. The lateral portal is shown at a higher magnification in the right cartoon. Here, the protein, as shown in the left cartoon, is slanted 30° towards the viewer to give a view down into the portal entrance. Panel B shows ATP-gated (30 µM) current and fluorescence traces of D331S mutant receptors recorded from two different cells in the presence and absence of IVM (10 µM). In the left traces, the current was evoked in the absence of IVM. In the right traces, the cell was pre-incubated in IVM for 5 min before re-applying ATP. Currents evoked in the presence of IVM deactivated slower and had reduced Pf%. Average values of Pf% are shown in Panel C. Panel D shows ATP-gated current and fluorescence traces of two different cells expressing the E51Q mutant receptor evoked in the absence (left traces) and presence (right traces) of IVM. Panel E shows the effect of 10 µM IVM on the kinetics of currents evoked by ATP (3 µM). Current was first tested in the absence of IVM (black). Then, IVM was applied for 5 min, and ATP was reapplied to evoke modified current (red).
Figure 5. Imparting IVM sensitivity through mutagenesis. The figure shows the effect of IVM on wt and double-mutant zfP2X4.1Rs. IVM (3 μM) increases peak amplitude of the ATP-gated (100 μM) current of the wt receptor without affecting the deactivation time course (A). The double-mutant zfP2X4.1R showed an altered rate of deactivation in the presence of IVM (B). Panel C shows the pooled data. zfDM is the N54E/N334D double charge mutant of zfP2X4.1R; rX2 DM is the Q52E/S326D double charge mutant of rP2X2R. Panel D shows the lack of effect of IVM on the double-mutant rP2X2R. ATP-gated currents were evoked in the absence (black trace) and presence (red trace) of IVM. The decrease in the peak current amplitude does not reflect an action of IVM because repeated ATP applications evoked in the absence of IVM show an identical decline (data not shown). The cause of the decline is unknown.
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