# Stargazin attenuates intracellular polyamine block of calcium-permeable AMPARs

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## 1 Recombinant receptors

### 1.1 Heterologous expression

Recombinant receptors were expressed in tsA201 cells (a gift from Raquel Yustos, Imperial College London). Cells were grown in DMEM (Sigma-Aldrich Co. Ltd., Gillingham, UK) supplemented with 10% FBS (GIBCO; Invitrogen Ltd., Paisley, UK), 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma) at 37°C, 5% $CO_2$ and maintained according to standard protocols. Transient transfection of receptor subunit combinations was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For homomeric receptors, cells were transfected at a ratio of 1:1 (AMPAR subunit:stargazin); for heteromeric receptors, the transfection ratio was 1:1:2 (subunit:subunit:stargazin). In each case, total DNA was 0.8 $\mu$g. Cells were split and plated on glass coverslips after 18–24 hours and recordings were made after a further 24–72 hours. Cells were maintained in 20 $\mu$M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Bioscience, Bristol, UK) to avoid AMPAR-mediated toxicity. AMPAR subunit DNAs (rat) were gifts from Stephen Heinemann (Salk) and Peter Seeburg (Heidelberg). Stargazin-EGFP DNA (mouse) was from Roger Nicoll (UCSF).

### 1.2 Electrophysiology

Cells were viewed with a fixed stage upright microscope (Axioskop FS1; Zeiss, Welwyn Garden City, UK) and EGFP-positive cells were selected for patch-clamp recording. Macroscopic currents were recorded at room temperature (22–24 °C) from outside-out membrane patches. Electrodes were fabricated from borosilicate glass (1.5 mm o.d., 0.86 mm i.d.; Harvard Apparatus, Edenbridge, UK) and had a resistance of 8–12 M$\Omega$. Recordings were made using an Axopatch 200A amplifier and acquired using a Digidata 1200 interface board and pClamp 7.1 or 8.0 software (Molecular Devices Corporation, Sunnyvale, CA). For experiments involving voltage jumps or rapid agonist application currents were low-pass filtered at 10 kHz and digitized at 20 or 50 kHz; for experiments involving ramp changes in membrane voltage currents were low-pass filtered at 1 kHz and digitized at 2 kHz.

### 1.3 Fast agonist application

To produce rapid solution changes at the excised patch, an application tool made from theta glass (2mm o.d.; Hilgenberg GmbH, Malsfeld, Germany) pulled to a tip opening of $\sim$200 $\mu$m, was

mounted on a piezoelectric translator (Burleigh LSS-3000/PZ-150M; EXFO Life Sciences & Industrial Division, Mississauga, Ontario or P-265.00, Physik Instrumente, Waldbronn, Germany). Control and agonist solutions flowed continuously through the two barrels and solution exchange occurred when movement of the translator was triggered by a voltage step (pClamp). For high frequency applications, glutamate (1 mM) was applied in 10 consecutive pulses of 1ms duration, at a frequency of 14 Hz with 10 $\mu$M spermine added to intracellular solution. For other experiments, glutamate pulses were usually of 100 ms duration (10 mM). At the end of each experiment, the adequacy of the solution exchange was assessed by destroying the patch and measuring liquid-junction current at the open pipette. Averaging open-tip responses from 29 experiments gave a 10–90% risetime of 96.7 $\pm$ 16 $\mu$s (mean $\pm$ s.e.m.).

## 1.4 Conductance-voltage relationships

To quantify the effects of stargazin on block by internal polyamines at different holding potentials, *I-V* plots were fit with 5–7$^{\text{th}}$ order polynomials; in all cases, the estimated reversal potential was close to 0 mV, and this value was used to generate conductance–voltage (*G-V*) plots. *G-V* curves were fitted (at negative voltages) to a Boltzmann function:

$$G = G_{\max}\left(\frac{1}{1+\exp((V_{\mathrm{m}}-V_{\frac{1}{2}})/k}\right) \tag{1}$$

where $G_{\max}$ is the maximal glutamate-activated conductance at hyperpolarized voltages, $V_{\frac{1}{2}}$ is the voltage at which spermine block is half-maximal and $k$ is a slope factor describing the membrane potential shift necessary to cause an *e*-fold change in conductance.

When comparing the effect of stargazin on the action of spermine and spermidine, *G-V* plots were fitted to a Woodhull model for an impermeable blocker:

$$K_{\mathrm{d}} = K_{\mathrm{d}(0)}\exp(-V_{\mathrm{m}}z\theta F/RT) \tag{2}$$

where $K_{\mathrm{d}(0)}$ is the apparent dissociation constant at 0 mV, $z$ is the valence of the polyamine, $V_{\mathrm{m}}$ is the membrane voltage, $\theta$ is the fraction of the membrane electric field experienced by the polyamine, and $R$, $T$ and $F$ have their usual meanings. Nonlinear least-squares fitting was done using the Levenberg-Marquardt algorithm (IGOR Pro 5.05; Wavemetrics Inc, Lake Oswego, OR).

For analysis of the concentration-dependence of block by spermine, *G-V* plots were obtained for a range of spermine concentrations and used to generate plots of normalized conductance ($G$) against spermine concentration ([Spm]) for each voltage. These inhibition curves were fit to the equation:

$$G = \frac{1}{1+\left(\frac{IC_{50}}{[\mathrm{Spm}]}\right)^{n_{\mathrm{H}}}} \tag{3}$$

where $IC_{50}$ is the concentration of spermine producing a half-maximal reduction in the conductance and $n_{\mathrm{H}}$ is the slope factor (Hill coefficient). For negative and positive voltages

independently, multiple datasets were fit simultaneously with a common value of $n_{\mathrm{H}}$ (Global Fit within IGOR Pro).

# 2 Cerebellar interneurons

## 2.1 Slice preparation

Coronal slices (200 $\mu$m) were made from the cerebellar vermis of P8 (7-8 day old), P18 (17-19 day old), and P28 Sprague-Dawley rats. Following decapitation, in accordance with UK Animals (Scientific Procedures) Act 1986, slices were cut with a moving blade microtome (DTK-1000; Dosaka EM Company, Kyoto, Japan) in ice-cold slicing solution containing (in mM): 85 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 4 $MgCl_2$, 25 $NaHCO_3$, 1.25 $NaH_2PO_4$, 64 sucrose, 25 glucose, and 0.02 D-2-amino-5-phosphonopentanoic acid (D-AP5; Tocris Bioscience). This was bubbled with 95% $O_2$ and 5% $CO_2$, pH 7.4. The slices were then kept at 32°C for 40 min. During the final 20 min of this period, the slicing solution was gradually exchanged with extracellular solution containing (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.25 $NaH_2PO_4$ and 25 glucose, also bubbled with 95% $O_2$ and 5% $CO_2$. Slices were then perfused with this solution at room temperature for a further 20 min before recording.

## 2.2 Recording and stimulation

Slices were viewed with a fixed stage upright microscope (Axioskop FS1) and whole-cell recordings were made from visually identified interneurons in the outer third of the molecular layer (presumptive stellate cells). Electrodes (3-6 MΩ; as above) were filled with a solution containing (in mM): 128 CsCl, 10 HEPES, 10 EGTA, 10 TEACl, 1 QX314 (Tocris), 2 $Mg_2$ATP, 1 $CaCl_2$, 2 NaCl, 0.25 D600 (Sigma) and 0.1 spermine, adjusted to pH 7.4 with CsOH, giving a final osmolarity of 285 $\pm$ 5 mOsmol/l. Whole-cell capacitance and series resistance were 5.67 $\pm$ 0.3 pF and 12.8 $\pm$ 0.5 MΩ, respectively ($n$ = 35). Series resistance was monitored continuously and the experiment was discarded if it increased by more than 30%.

# 3 Free polyamine concentration

## 3.1 Spermine

The concentrations of free spermine ($[\mathrm{Spm}]_{free}$) were calculated using published affinity coefficients for the binding of spermine by ATP and Mg:ATP [1]. These were inverted to give the dissociation constants: $K_{\mathrm{d}}1 = 1.44 \times 10^{-4}$ M, $K_{\mathrm{d}}2 = 9.43 \times 10^{-4}$ M, $K_{\mathrm{d}}3 = 3.53 \times 10^{-4}$ M and $K_{\mathrm{d}}4 = 3.70 \times 10^{-4}$ M; where:

$$[\mathrm{ATP}] = \frac{K_{\mathrm{d}}1[\mathrm{ATP} : \mathrm{Mg}]}{[\mathrm{Spm}]_{free}} \tag{4}$$

$$[\mathrm{Mg} : \mathrm{ATP}] = \frac{K_{\mathrm{d}}2[\mathrm{Spm} : \mathrm{Mg} : \mathrm{ATP}]}{[\mathrm{Spm}]_{free}} \tag{5}$$

$$[\mathrm{Spm:ATP}] = \frac{K_\mathrm{d}3[\mathrm{Spm:Mg:ATP}]}{[\mathrm{Mg}]_{free}} \tag{6}$$

$$[\mathrm{ATP}] = \frac{K_\mathrm{d}4[\mathrm{Spm:ATP}]}{[\mathrm{Spm}]_{free}} \tag{7}$$

and

$$[\mathrm{Spm}]_{free} = [\mathrm{Spm}]_0 - [\mathrm{Spm:Mg:ATP}] - [\mathrm{Spm:ATP}] \tag{8}$$

$$[\mathrm{Mg}]_{free} = [\mathrm{Mg}]_0 - [\mathrm{Spm:Mg:ATP}] - [\mathrm{Mg:ATP}] \tag{9}$$

In a closed system or cycle, multiplying the dissociation constants in one direction should equal the dissociation constants in the other. So:

$$\frac{K_\mathrm{d}1K_\mathrm{d}2}{[\mathrm{Mg}]_{free}[\mathrm{Spm}]_{free}} = \frac{K_\mathrm{d}3K_\mathrm{d}4}{[\mathrm{Mg}]_{free}[\mathrm{Spm}]_{free}} \tag{10}$$

Accordingly, we used a slightly altered value of $K_\mathrm{d}3 = 3.85 \times 10^{-4}$ M to close the system.

By rearranging and combining equations 5, 6, 8 and 9 above it is possible to create a quadratic equation describing the value of $[\mathrm{Spm}]_{free}$ in terms of $[\mathrm{Mg}]_{free}$:

$$[\mathrm{Spm}]_{free}{}^2 + [\mathrm{Spm}]_{free}\left(K_\mathrm{d}2 - [\mathrm{Spm}]_0 + ([\mathrm{Mg}]_0 - [\mathrm{Mg}]_{free})\left(\frac{K_\mathrm{d}3}{[\mathrm{Mg}]_{free}} + 1\right)\right) - [\mathrm{Spm}]_0 K_\mathrm{d}2 = 0 \tag{11}$$

The value of $[\mathrm{Mg}]_{free}$ in Eq. 11 was then determined using 'Solver' within Excel (Microsoft, Redmond, WA) so that equations 4 to 9 were simultaneously satisfied.

### 3.2 Spermidine

Given that a similar set of rate constants has not been published for spermidine, we used a different calculation based on a global affinity constant for total ATP with or without $\mathrm{Mg}^{2+}$ ($5.59 \times 10^2$ $\mathrm{M}^{-1}$ [1]), giving $K_\mathrm{d}$Spd = $1.79 \times 10^{-3}$ M, where:

$$[\mathrm{ATP}] = \frac{K_\mathrm{d}\mathrm{Spd}([\mathrm{Spd}]_0 - [\mathrm{Spd}]_{free})}{[\mathrm{Spd}]_{free}} \tag{12}$$

This can be rearranged to the quadratic equation:

$$[\mathrm{Spd}]_{free}{}^2 + ([\mathrm{Spd}]_0 + [\mathrm{ATP}]_0 + K_\mathrm{d}\mathrm{Spd})[\mathrm{Spd}]_{free} - K_\mathrm{d}\mathrm{Spd} = 0 \tag{13}$$

This gave a free concentration of 31.3 $\mu$M spermidine for 100 $\mu$M added.

# References

[1] Meksuriyen, D. *et al.* Formation of a complex containing ATP, $Mg^{2+}$, and spermine. Structural evidence and biological significance. *J Biol Chem* **273**, 30939–30944 (1998).