Investigating the Binding Site of Positive Allosteric Modulators of P2X Receptors

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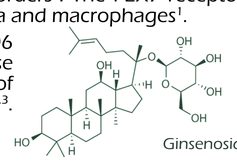
Introduction

P2X receptors are ligand-gated ion channels activated by adenosine 5'-triphosphate (ATP). There are seven different subunits of P2X receptor, termed P2X1 through to P2X7. Each P2X receptor is made up of three of these subunits to form a trimeric structure. These subunits can be identical (homomeric trimers) or a mixture of subunits (heteromeric trimers).

P2X receptors have numerous, often not fully understood, roles throughout the body. The P2X2 receptor is thought to have a role in vestibular function and perhaps even depression[1]. The P2X4 receptor is involved with neuropathic pain and cardiac function, and recent research has shown that it may be a useful target for alcohol use disorders[1]. The P2X7 receptor is involved in cell death, but also helps to regulate the immune response, particularly in microglia and macrophages[1].

Clinical trials are being conducted into antagonists of some P2X receptors (see NCT04116606 and NCT01432730), but there may also be a role for positive allosteric modulators of these receptors in patients with defective responses. Recently, a novel binding pocket for a number of positive allosteric modulators derived from ginseng has been identified in P2X7 and P2X4[2,3]. Here, the binding pocket is being further investigated at the P2X2 and P2X4 receptors.



Ginsenoside CK

Materials and Methods

Cell Culture

HEK-293 cells stably transfected with hP2X2a, hP2X4 or hP2X4 were maintained in DMEM/F12 media containing L-glutamine and supplemented with 10% FBS, 10000U mL^{-1} penicillin, 10mg mL^{-1} streptomycin, and 400 µg mL^{-1} G418. Non-transfected HEK-293 cells were maintained in the same media without G418 prior to transient transfection. All cells were maintained at 37°C with 5% CO2 in a humidified incubator.

Mutagenesis and Transfection

Point mutations were introduced into the WT hP2X2a and hP2X4 plasmids using the Stratagene Quikchange-II Site-Directed Mutagenesis kit. PCR was performed using PFU Ultra High-Fidelity DNA polymerase and products digested with DPN1 for 1 hour at 37°C. NEB® 5-alpha F'Iq Competent E. coli High Efficiency cells (C2992, New England Biolabs, UK) were transformed using 5cL of digested PCR product following the NEB High Efficiency Transformation Protocol . Transfections were performed using Lipofectamine 2000 and cells were tested 48 hours post-transfection.

Membrane Potential Assay

Dose responses to ATP were conducted on HEK-293 cells stably expressing P2X2, P2X4 and P2X7 using the Membrane Potential Blue kit (Molecular Devices) and a Flexstation 3 Multimode Plate Reader (Molecular devices). Cells were plated onto poly-D-lysine coated 96 well plates at $2x10^4$ cells per well the day prior to experimentation. Experiments were carried out in standard extracellular buffer (145mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$,13mM glucose, 10mM HEPES, pH 7.3-7.4). Data were collected using SoftMax Pro v5.4 and analysed using GraphPad Prism v8.

Dose responses to ATP in stably transfected HEK-293

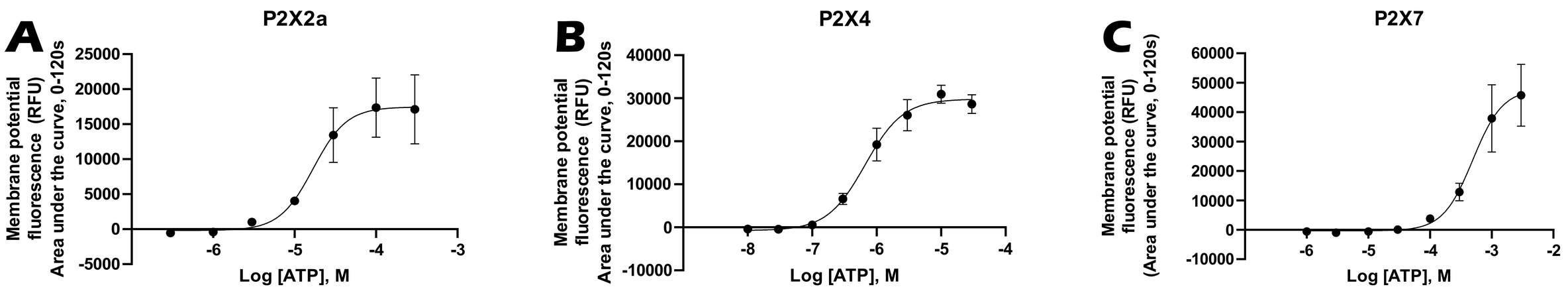


Figure 1. Dose responses to ATP in HEK-293 cells stably expressing P2X2a, P2X4, and P2X7 receptors. A: ATP dose responses in stably transfected HEK-P2X2a. EC_{50}16.89 µM (n=4). **B:** ATP dose responses in stably transfected HEK-P2X4. EC_{50} 0.6626 µM (n=6). **C:** ATP dose responses in stably transfected HEK-P2X7. EC_{50} 497.2mM (n=3).

Simulated docking of ginsenoside CK

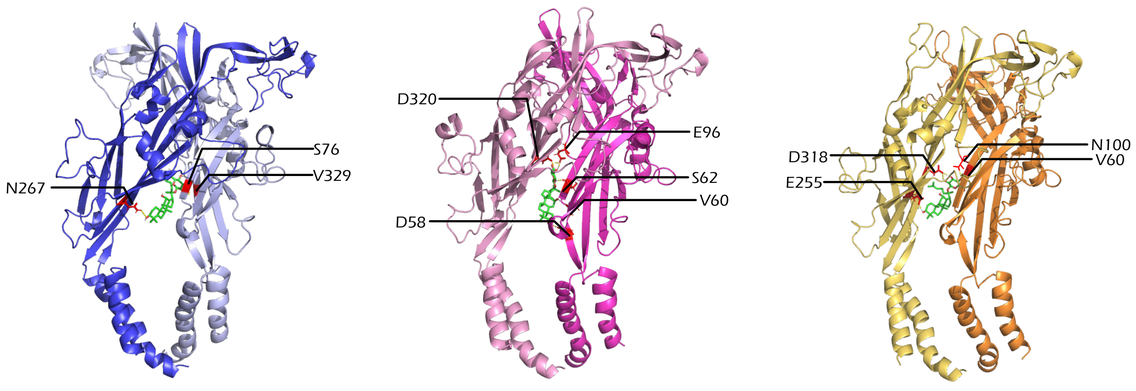


Figure 2. Induced fit docking of ginsenoside CK at P2X2a (blue), P2X4 (pink), and P2X7 (yellow) with proposed interacting residues highlighted in red. All three receptors show CK docked within the central vestibule region, suggesting a conserved binding site. In P2X2, CK appears to interact with the left side of the central vestibule. In P2X4, CK appears to interact with the right side. In P2X7, binding occurs higher up in the central vestibule region.

Binding pocket

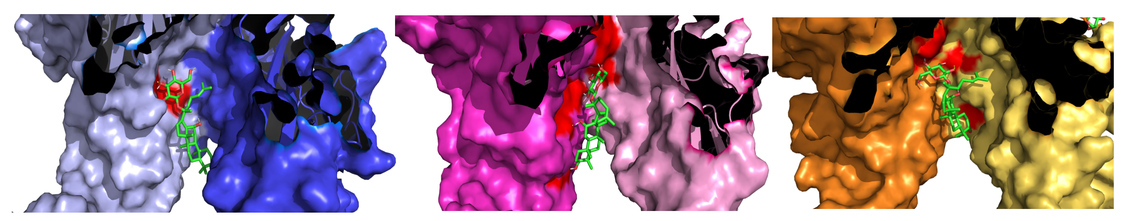


Figure 3. Close up of the proposed binding site of ginsenoside CK in P2X2a, (blue), P2X4 (pink), and P2X7 (yellow) with mutated residues highlighted in red. In P2X2 and P2X7, the sugar can bee seen interacting with the top left of the central vestibule in a similar orientation. The side chain is also interacting with the top of the pocket in these receptors. In P2X4, the sugar is rotated approximately 90 degrees, but still interacts with the upper central vestibule region. The steroid scaffold of ginsenoside CK can be seen to hang lower in the pocket in P2X2 and P2X4, with the C3 hydroxyl group interacting with N267 (P2X2a) and D58 (P2X4) respectively. In P2X7, the steroid scaffold appears more compact, with the C3 hydroxyl interacting with E255.

Effects of binding pocket mutations in hP2X2a

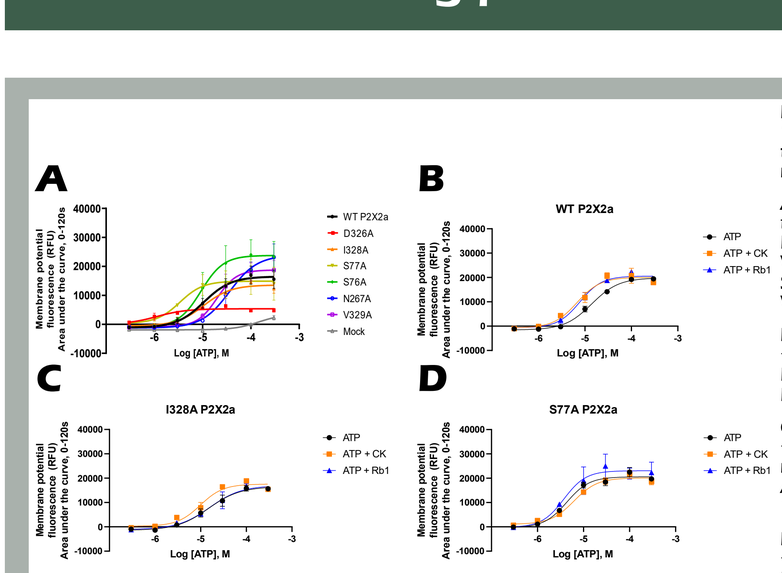


Figure 4. Dose responses to ATP, ATP + 10µM CK, and ATP + 10µM Rb1 in transiently transfected wild-type and mutant HEK-P2X2a.

A: Dose responses to ATP in a range of transiently transfected mutant and wild-type HEK-P2X2a (n=2-4). EC_{50} for each as follows: **WT:**10.6µM **D326A:**11.3µM **I328A:**10.6µM **S77A:**3.4µM **S76A:**9.3µM **N267A:**36.2µM **V329A:**19.8µM **Mock:** 145.0mM

B: Dose responses to ATP, ATP + CK and ATP + Rb1 in transiently transfected WT HEK-P2X2a (n=1). ATP EC_{50}: 14.0µM ATP + CK EC_{50}: 6.7µM ATP + Rb1 EC_{50}: 7.8µM

C: Dose responses to ATP, ATP + CK and ATP + Rb1 in transiently transfected I328A mutant HEK-P2X2a (n=1). ATP EC_{50}: 14.8µM ATP + CK EC_{50}: 9.8µM ATP + Rb1 EC_{50}: 15.7µM

D: Dose responses to ATP, ATP + CK and ATP + Rb1 in transiently transfected S77A mutant HEK-P2X2a (n=1). ATP EC_{50}: 4.3µM ATP + CK EC_{50}: 6.2µM ATP + Rb1 EC_{50}: 3.7µM

Effects of binding pocket mutations in hP2X4

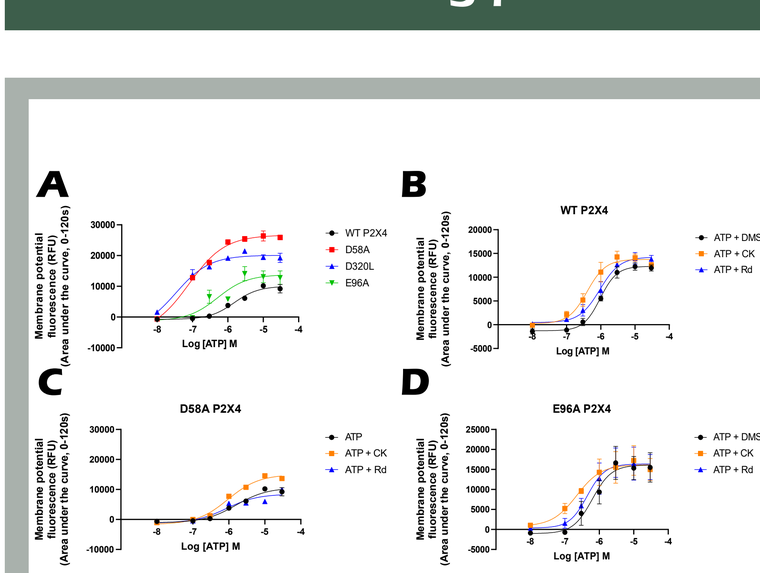


Figure 5. Dose responses to ATP, ATP + 10µM CK, and ATP + 10µM Rd in transiently transfected wild-type and mutant HEK-P2X4.

A: Dose responses to ATP in a range of transiently transfected mutant and wild-type HEK-P2X4 (n=1-3). EC_{50} for each as follows: **WT:**1.5µM **D58A:** 79.8 nM **D320L:** 33.9nM **E96A:** 0.52µM

B: Dose responses to ATP, ATP + CK and ATP + Rd in transiently transfected WT HEK-P2X4 (n=4). ATP EC_{50}: 0.96µM ATP + CK EC_{50}: 0.40µM ATP + Rb1 EC_{50}: 0.93µM

C: Dose responses to ATP, ATP + CK and ATP + Rd in transiently transfected D58A mutant HEK-P2X4 (n=1). ATP EC_{50}: 1.6µM ATP + CK EC_{50}: 0.94µM ATP + Rb1 EC_{50}: 0.85µM

D: Dose responses to ATP, ATP + CK and ATP + Rd in transiently transfected E96A mutant HEK-P2X4 (n=2). ATP EC_{50}: 0.63µM ATP + CK EC_{50}: 0.22µM ATP + Rb1 EC_{50}: 0.45µM

Conclusions

It appears that, similarly to P2X4 and P2X7, P2X2 can also be potentiated by ginsenosides such as CK. This suggests that the previously identified novel binding pocket in the central vestibule is conserved across P2X receptor subtypes. The extent to which ginsenosides are able to potentiate these receptors varies between the subunits, suggesting that there is some degree of difference between the pockets. These differences may allow for the development of selective positive allosteric modulators for each receptor type. These selective positive allosteric modulators could be used to target specific illnesses related to a particular P2X receptor without binding to other P2X receptors. Certain mutations appear to reduce the activity of ginsenosides at P2X2 and P2X4 receptors: I328A in P2X2 abolishes the potentiation by ginsenoside Rd, but is still potentiated by CK, suggesting that this residue is key to the action of CK. S77A in P2X2 does not appear to be potentiated by either ginsenoside, but the receptor itself is more sensitive to ATP than the wild type, suggesting that this residue could be important in receptor gating. More extensive mutagenesis around the binding pocket and other binding assays, such as FRET, may need to be investigated in future to confirm the presence of the conserved binding pocket.

References
1. Stokes L, Bidula S, Bibic L, Allum E. To inhibit or enhance? Is there a benefit to positive allosteric modulation of P2X receptors? Front. Pharmacol. 2020. (Accepted)
2. Bidula SM, Cromer BA, Walpole S, Angulo J, Stokes L. Mapping a novel positive allosteric modulator binding site in the central vestibule region of human P2X7. Sci Rep. 2019;9(1):3231.
3. Dhuna K, Felgate M, Bidula SM, Walpole S, Bibic L, Cromer BA, et al. Ginsenosides Act As Positive Modulators of P2X4 Receptors. Molecular pharmacology. 2019;95(2):210-21.