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Chapter 19

Heterologous expression and patch-clamp recording of P2X receptors in HEK293 cells

Lin-Hua Jiang and Sébastien Roger

Running head: P2X electrophysiology in HEK293 cells

Summary

P2X receptors (P2XRs) are ligand-gated ion channels gated by extracellular adenosine 5'-triphosphate (ATP) and play a critical role in mediating ATP-induced purinergic signaling in physiological and pathological processes. Heterologous expression of P2XR in human embryonic kidney 293 (HEK293) cells and measurement of P2XR-mediated currents using patch-clamp recording technique have been widely used to study the biophysical and pharmacological properties of these receptors. Combination of electrophysiology with site-directed mutagenesis and structural information has shed light on the molecular basis for receptor activation and mechanisms of actions by receptor antagonists and modulators. It is anticipated that such methodologies will continue helping us to provide more mechanistic understanding of P2XRs and to test novel receptor antagonists and allosteric modulators for therapeutical purposes. In this chapter, we describe protocols of transiently or stably expressing the P2XR in HEK293 cells and measuring P2XR-mediated currents by using whole-cell recording.

Key Words: P2X receptors; HEK293 cells; heterologous expression; patch-clamp recording

1. Introduction

P2X receptors (P2XRs) for extracellular ATP comprise the ionotropic P2 purinergic receptor family. There are seven different subunits, P2X1 to P2X7, which can assemble in homo-/hetero-trimeric ligand-gated ion channels gated by ATP as being the physiological agonist ***(1)***. P2XRs show wide expression in mammalian cells, both excitable and non-excitable cells, and have an important role in mediating ATP-induced purinergic signaling in a diversity of physiological processes ranging from neuromodulation ***(2)***, immune response ***(3)*** to regulation of stem cell functions ***(4, 5)***. Compelling evidence from both preclinical and clinical studies supports critical engagement of P2XRs in the pathogenesis of numerous diseases, including chronic pain ***(6)***, neurodegenerative diseases ***(7)***, mood disorders ***(8)***, inflammatory diseases ***(9)***, metabolic disorders ***(10)*** and cancers ***(11, 12)***.

Since its invention ***(13)***, the patch-clamp recording technique has been the golden experimental tool in the study of ion channels. Studies using this technique of the P2XRs recombinant in heterologous expression cell systems have revealed distinctive biophysical and pharmacological properties of these receptors, which has been useful in elucidating their physiological and pathological roles ***(1)***. Human embryonic kidney (HEK) 293 cells express no endogenous P2XR and, in addition, THEY are readily transfected with plasmids with good transfection efficiency, and robust in protein expression, membrane trafficking and post-translational modifications. The membrane of HEK293 cells is also amenable to formation of seal with patch-clamp electrodes. Therefore, HEK293 cells represent a widely used heterologous mammalian cell expression system to express the mammalian P2XRs. Electrophysiological studies of the P2XRs expressed in HEK293 cells, in combination with site-directed mutagenesis and more recently with structural information, have shed light on the ion-permeating pore ***(14)***, ATP binding ***(15-16)*** and conformational changes accompanying channel gating ***(17-22)*** as well as residues coordinating the actions of

antagonists and modulators ***(23-26)***. As have been nicely summarized in recent reviews ***(27-30)***, electrophysiological studies using HEK293 cells and other heterologous expression cell systems, such as *Xenopus* oocytes, have provided substantial insights into the molecular basis that determine ATP-induced activation of the P2XRs and actions of P2XR antagonists and allosteric modulators. In addition, measurements of agonist-induced currents in HEK293 cells expressing P2XR carrying disease-associated mutations have been helpful in informing the mutational effects on receptor functions and thereby furthering our understanding of the disease mechanisms ***(31-33)***. Electrophysiology using HEK293 cells as a mammalian cell expression system will continue to be a very useful tool to develop a better understanding of the P2XRs, particularly to test novel receptor antagonists and allosteric modulators for therapeutical purposes ***(8)***.

In this chapter, we describe protocols of using HEK293 cells to transiently and stably express P2XRs and using whole-cell patch-clamp recording to measure P2XR-mediated currents.

2. Materials

2.1 Cells and reagents

1. HEK293 cells (American Type Cell Collection).
2. Culture medium: Dulbecco's modified Eagles medium (DMEM)/F-12, with penicillin and streptomycin and 10 % foetal bovine serum (FBS).
3. Dulbecco's Ca^{2+}-/Mg^{2+}-free phosphate buffer saline (D-PBS)
4. 0.05% trypsin-EDTA solution
5. 400 μg/ml G418 in culture medium
6. Opti-MEM I serum free medium (Invitrogen), or similar.
7. Lipofectamine 2000 transfection reagent (Invitrogen), or similar.
8. Plasmids encoding wild-type (WT) and mutant P2XRs, generated in-house, most often using pcDNA3.1 vector (Invitrogen) or similar.

2.2 Equipment

1. Bench-top pH metre.
2. Advanced Instruments Osmo1, single sample micro-osmometer.
3. Sterile cell culture plastics: T25 vented flasks, 6-well and 96-well plates; 35-mm petri-dishes; 5-mL, 10-mL and 25-mL plastic pipettes; 15-mL conical centrifuge tubes; 1.5-mL microcentrifuge tubes.
4. 2-, 10-, 20-, 200- and 1000-μL pipettes and tips.
3. Bench top swing-out centrifuge.
4. Haemocytometer.
5. 13-mm glass coverslips.
6. CO_2 tissue/cell incubator.
7. 50-mL and 10-mL syringes.

8. 0.22-μm-pore diameter filters.

9. Borosilicate glass capillaries with 1.5-mm outer diameter and 1.12-mm inner diameter

10. AgCl-coated Ag pellet reference electrode.

11. PP-830 glass micropipette puller (see **Note 1**).

12. PC computer with 24-inch thin film transistor monitor.

13. Axopatch 200B patch-clamp amplifier, and 1332A Digidata (Molecular Devices) (see **Note 2**).

14. Data acquisition and analysis software: pClamp (Clampex and Clampfit; Molecular Devices), and Origin (OriginLab).

15. Inverted microscope (we use Axiovert-200 from Zeiss) (see **Note 3**).

16. MP-85 manual micro-manipulator (see **Note 4**).

17. RSC-160 rapid solution changer (see **Note 5**).

2.3. *Recording Solutions*

Prepare recording solutions with deionized water, adjust to pH 7.3, and measure the osmolarity. Store extracellular solutions at 4°C and warm to room temperature before use. Filter intracellular recording solution using a 50-mL syringe with a 0.22-μm-pore diameter filter attached and aliquot in 1-ml volume and kept at -20°C. Frost one aliquot of intracellular solution and thawed to room temperature before use.

1. Standard extracellular recording solution: 147 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 13 mM D-glucose, 10 mM HEPES; pH 7.3 with NaOH; ~300 mOsm (see **Note 15**).
2. Standard intracellular solution: 145 mM NaCl, 10 mM EGTA and 10 mM HEPES; pH 7.3 with NaOH; ~290 mOsm (see **Note 16**).

3. 100 mM ATP and its analogues (e.g., BzATP) in water as stock solutions. Adjust ATP stock solution to pH 7.3 with NaON if used in mM to elicit P2X7R activation. Aliquot ATP and BzATP stock solutions in small volumes, and store at -20°C. Prepare working solutions with desired concentrations by diluting the stock solution in extracellular recording solutions.

3. Methods

Carry out the following procedures for cell culture, transfection and cell plating in a tissue culture fume hood at room temperature. Maintain HEK293 cells in culture medium (see **Note 6**) in a tissue culture incubator at 37°C and 5% CO_2 under humidified conditions. Passage HEK293 cells or P2XR-expressing stable cells every 3-4 days or when cell confluency reaches ~80%. Warm all culture media to room temperature (20-22°C) before use.

3.1 Cell Passage

1. Remove media and rinse cells with 1 ml D-PBS. Add 1-2 ml trypsin-EDTA, and incubate cells at 37°C for 1-2 min (see **Note 7**).
2. Once cells are detached, add 1-2 ml fresh culture medium, and transfer the cell suspension in a 15-ml conical centrifuge tube.
3. Collect cells by centrifugation using a bench-top centrifuge at 1000 rpm for 5 min.
4. Discard the supernatant, re-suspend the cell pellet gently and thoroughly in 2 ml fresh culture medium (see **Note 8**). Transfer 10-20% of cells (0.2-0.4 ml) to a new T25 flask, depending on the confluency of starting cell cultures, frequency of using cells and cell proliferation.

3.2 Transient transfection

1. For each transfection, seed ~10^6 cells for one well in a 6-well plate or a 35-mm petri-dish and incubate cells overnight or until reaching 70-80% confluency.
2. For each transfection, dilute 1 μg plasmid encoding P2XR (and 0.1 μg plasmid encoding enhanced green florescence protein (eGFP) for transient expression of P2XR; see **Note 9**) in 100 μl Opti-MEM medium in one 1.5-ml microcentrifuge tube, and 3 μl

Lipofectamine 2000 into 100 µl Opti-MEM medium in a second tube. Incubate them for 5 min at room temperature.

3. Combine the contents into one single tube, and incubate further 20 min.
4. Add 800 µl fresh culture medium into the tube, and mix the content.
5. Remove media from cell-containing well or petri-dish, and replace with the transfection medium (see **Note 10**).
6. Return cells to the CO_2 incubator, and incubate for 24-48 hr before use for patch-clamp recording or 48 hr for generating stable cell lines (see **Note 11**).

3.3 Generation of stable cell lines expressing P2XR

1. Following the steps described above in section 3.2, replace the transfection medium with 1 ml fresh culture media containing G418 antibiotic used as the selecting agent when using expression vectors such as pcDNA3.1 bearing the G418 resistance gene.
2. Culture the cells for 1-2 weeks, with replacing G418 every 2-3 days.
3. Treat the cells with 1 ml trypsin-ETDA, plate individual islands of cells in separate wells in 96-well plates, and incubate the cells until confluent in the presence of G418 (see **Note 12**).
4. Detach cells in each well with 100 µl trypsin-ETDA, transfer cells to T25 flasks, and grow cells in 5 ml fresh culture medium until confluent.
5. Validate stable P2XR-expressing cells by plating cells on coverslips and measuring agonist-induced currents using patch-clamp recording described below (see **Note 13**).

3.4 Cell Plating for Electrophysiological Recordings

1. To prepare cells for recording, remove transfection medium (for transient expression) or culture media, wash with D-PBS, and detach cells using trypsin-EDTA as described above.
2. Place 13-mm glass coverslips (up to 4) per well in a 6-well plate or per 35-mm petri-dish, and add 1 ml fresh culture medium in one well or petri-dish.
2. Count cell number using a haemocytometer. Transfer 40,000 cells per well or petri-dish, and incubate cells for 12-24 hr before use (see **Note 14**).

3.5 Whole-Cell Recordings

1. Switch on the patch-clamp rig, and open Clampex for recording and RSC software that controls solution changes.
2. Connect the recording chamber to solution reservoir provided by a 50-mL syringe, add extracellular recording solution into the syringe, and adjust solution flow at a rate of 1-2 ml min^{-1}.
3. Connect the recording chamber to RSC solution tubes provided by 10-mL syringes. Add agonists, antagonists, or other agents into syringes, and register them in the RSC control system.
4. Check AgCl-pellet reference electrode in contact with extracellular recording solution and connection to the ground via the headstage of the amplifier.
5. Prepare recording pipettes from glass capillaries using PP-830 puller. Back-fill the pipette with intracellular recording solution, and mount it onto the holder of the headstage that is connected to the amplifier via AgCl-coated silver wire (see **Note 17**).
6. Lower the pipette into the extracellular solution in the recording chamber, and apply a 5 mV test pulse to determine the resistance of the pipette, which should be in the range of 3-5 MΩ.

7. Identify single cells under the microscope (see **Note 18**).

8. Adjust the basal current level to zero. Manoeuvre the pipette using a micro-manipulator under the microscope to bring its tip into contact with cell surface, which is indicated with slightly increased resistance of the pipette.

9. Apply suction to the cell membrane, through a syringe connected via a tube to the recording pipette, to form a gigaohm seal (in cell-attached configuration). Compensate the transient capacitive currents (refer to the amplifier's manuscript for details).

10. Apply additional suction to break-through the cell membrane to achieve whole-cell configuration, which is indicated by appearance of relatively slow capacitive currents (see **Note 19**). Compensate the capacitive currents. Set the membrane potential to -60 mV or desired holding potential (see **Note 20**).

11. Compensate the capacitive current by ~60-70% (see **Note 21**).

12. Start recording agonist-induced P2XR-mediated currents. Apply agonists for 2 to 10 s through RSC at an interval of 2-4 min, depending on the activation and desensitization properties of the P2XR under investigation, which requires optimization experimentally. The following are some commonly used protocols in the study of the P2XRs:

13. To determine the potency or EC_{50} value of an agonist by constructing the concentration-activation relationship curve, apply agonist at 2-4 min intervals from low to high concentrations (see an example in Fig. 1).

14. To determine the potency or IC_{50} value of an antagonist at P2XR by constructing the concentration-inhibition relationship curve, chose the concentration of agonist to elicit measureable currents (e.g., EC_{50} or $EC_{90\text{-}100}$), apply agonist at 2-4 min intervals to establish stable current response, and treat the patched cell with antagonist between agonist applications at increasing concentrations (see an example in Fig. 2). For the

antagonists with fast dissociation kinetics, co-apply antagonist with agonist as well as treatment with antagonist between agonist applications (see example in Fig. 3).

15. To examine the effect of exposure to reagent, for example, dithiothreitol (DTT) used in cysteine substitution studies, on P2XR-mediated currents. Apply agonist at a chosen concentration at 2-4 min to establish the stable current response before exposing the patched cell with DTT between agonist applications (see an example in Fig. 4).

4. Notes

1. PP-830 is discontinued and replaced by PC-100. Other models of micropipette pullers from different vendors can be used.
2. Molecular Devices provides Axopatch 200B amplifier, which offers low-noise recordings, particularly suitable for single-channel recording, and MultiClamp 700B amplifier (https://www.moleculardevices.com/products/axon-patch-clamp-system/amplifiers/axon-instruments-patch-clamp-amplifiers). Harvard Bioscience provides HEKA EPC 10 USA amplifier (https://www.heka.com/products/products_main.html#physiol_epc10single). These are the most common models.
3. Several manufactures provide inverted microscopes that are suitable for building a manual patch-clamp rig. A fluorescence system incorporated into the microscope is required to identify eGFP-positive cells, if cells are co-transfected with plasmids for P2XR and eGFP.
4. There are many choices of manual and automatic micromanipulators.
5. A computer-controlled rapid solution changing system is highly desired for application of agonists for a few seconds to P2XR to avoid receptor desensitization. Several manufacturers provide fast solution change systems.
6. Supplementing the culture medium with antibiotics is not essential, but it is recommended to add 50 units/mL penicillin and 50 μg/mL streptomycin to prevent contamination, if required.
7. Avoid prolonged incubation with trypsin-EDTA, particularly when plating cells for recording. Apply gentle taping of the flask, plate or petri-dish from side or bottom can help to dislodge loosely-attached cells.
8. Use a 1000-μl pipette to pipette up and down 10-20 times and, if required, then a 200-μL pipette to pipette up and down 10-20 times to break down cell clumps to single cells.

9. The efficiency of transfection has been improved using commercially available reagents but falls far away from 100%. Therefore, including 0.1 μg plasmid encoding eGFP during transfection is highly recommended, if transfected cells are used for recording and the microscope in the patch-clamp rig has a fluorescence system. To select eGFP-positive cells post-transfection for recording.
10. The protocol in detail using Lipofectamine2000 reagent to transiently transfect the cells is available at: https://www.ecu.edu/cs-dhs/biochemistry/upload/Transfection-Protocol.pdf). Several other transfection reagents or kits are also suitable for transient expression.
11. Optional: if 48 hr incubation time is preferred, replace transfection medium with culture medium 24 hr post-transfection, and incubate cells further 24 hr before use.
12. Repeat this sub-cloning step, if necessary.
13. Other methods can be used to validate the stable cell lines, such as measurement of agonist-induced calcium responses, or through molecular biology and biochemical techniques (e.g., RT-PCR, western blotting).
14. Cells can be used for patch-clamp recording after shorter incubation times, if cells adhere to coverslip faster or earlier. In addition, plate cells at lower cell density if cells are used 36-48 hr post-transfection.
15. P2X7Rs are inhibited by extracellular Ca^{2+} and Mg^{2+}. Experiments recording P2X7R-mediated currents often use extracellular low divalent cation solution: 147 mM NaCl, 2 mM KCl, 0.3 mM $CaCl_2$, 23 mM D-glucose, 10 mM HEPES, pH 7.3 with NaOH; ~300 mOsm. Please note that it is possible but difficult to form gigaohm seal in low divalent cation solution. It is recommended to establish the gigaohm seal or whole-cell configuration in standard extracellular solution and then change to low divalent cation solution.

16. For not fully understood reasons, fluoride ions in the intracellular solution can improve seal formation and stabilise the cell membrane, favouring longer and more stable recordings. Replace NaCl in the intracellular solution in part or whole with NaF, if required.
17. Poor chloride coating of the silver wire can result in a drift of the basal or zero current level over the time. Coat the silver wire regularly in bleach for 30-60 min, rinse with water, and dry before use.
18. As mentioned above, if HEK293 cells are transiently co-transfected with eGFP and P2XR, a fluorescence microscope is required to identify eGFP-positive cells.
19. If it is difficult to establish the whole-cell configuration following seal formation, use the "zap" button on the front panel of the amplifier to rupture the cell membrane. However, this practice is not recommended for routine use, as zapping often reduces or lose the sealing.
20. Holding the cell membrane at a negative potential can help stabilize or improve the sealing.
21. Determine the series resistance and cell capacitance. The cell capacitance can be used to derive the current density, which can mitigate the effect of the cell size on the current amplitude (see an example in Fig. 1). Check the series resistance during the recording and use data with a series resistance of no more than 10 MΩ.

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5. References

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Figure Legends

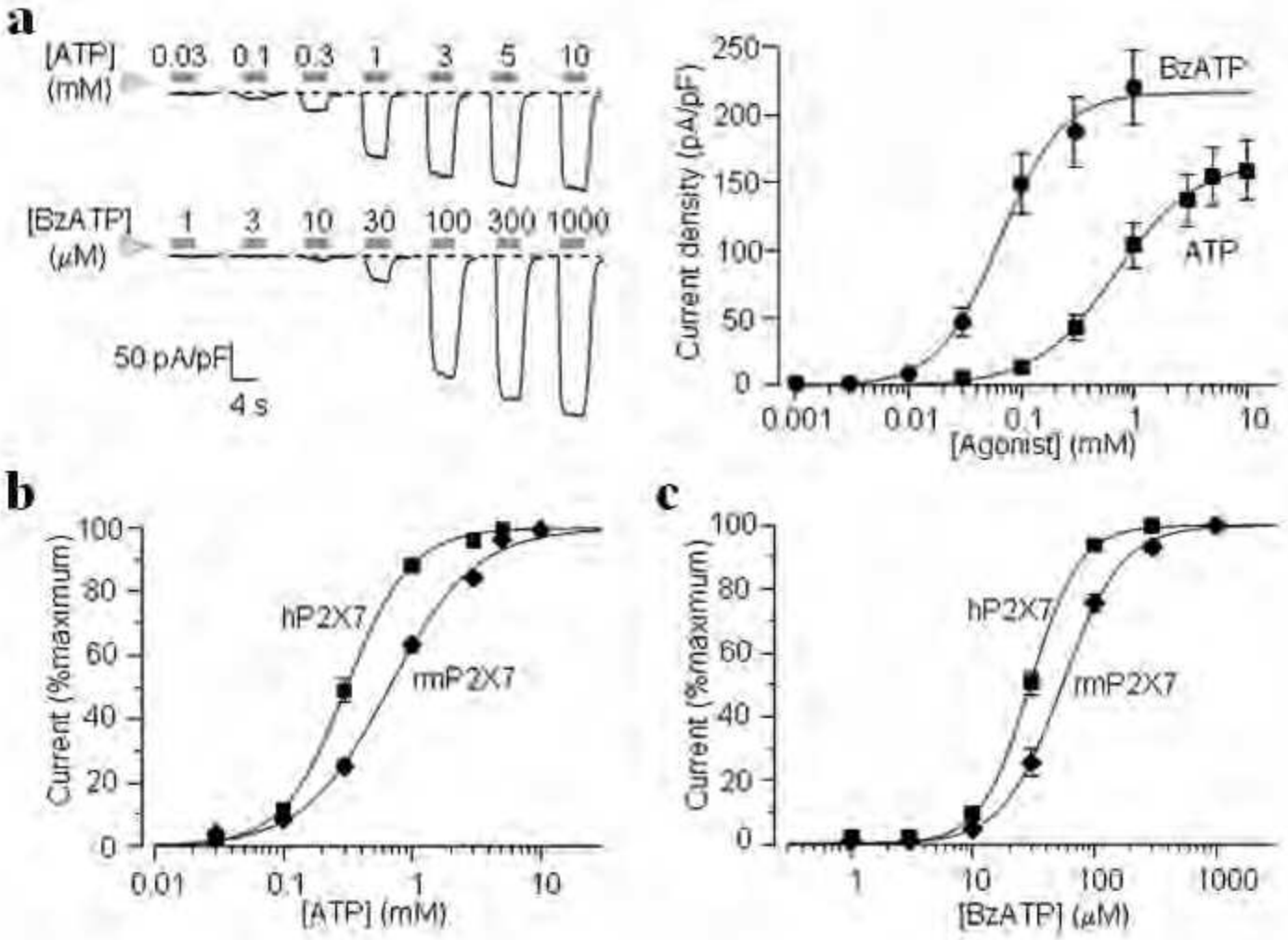
Figure 1. Comparison of the sensitivity of rhesus macaque (rm) and human P2X7Rs to ATP and BzATP. **(a)** *Left*, representative currents evoked by different concentrations of ATP or BzATP in HEK293 cells expressing rmP2X7R. *Right*, agonist concentration-current curves. Each data point represents mean from 8-11 cells for ATP and 4-6 cells for BzATP. **(b-c)** Comparison of agonist concentration-current relationship curves between rmP2X7R and hP2X7R. The data for rmP2X7R are from panel (a). Each data point represents mean from 12 cells for ATP (b) and 5 cells for BzATP (c) for hP2X7R. The smooth curves show the fit of the mean data to the Hill equation. Take from ***(34)***.

Figure 2. Inhibition of hP2X7R by a novel antagonist. (a) Representative currents from a HEK293 cell expressing hP2X7R before (CTL) and after treatment with compound C23 at different concentrations. Currents were elicited in extracellular low divalent cation solution by 4-s application of 300 μM BzATP at an interval of 2-4 min. Current inhibition was reversed upon washing. **(b)** Antagonist concentration-current inhibition relationship curves. Each data point represents mean from 4 cells. The smooth line shows the fit of the mean data to the Hill equation. Modified from ***(35)***.

Figure 3. Inhibition of rhesus macaque and human P2X7Rs by A-438079. (**a**) Representative ATP-induced currents in HEK293 cells expressing rmP2X7R or hP2X7R in the absence (control) or presence of different concentrations of A-438079. Currents were evoked by 4-s application of 3 mM ATP at rmP2X7R and 1 mM at hP2X7R every 4 min. Cells were exposed to A-438079 between and during ATP applications. (**b**) Comparison of antagonist concentration–current inhibition curves

between rmP2X7R and hP2X7R. The smooth lines show the fit of the mean data to the Hill equation. Modified from ***(34)***.

Figure 4. DTT-induced reversible increase in BzATP-induced currents in HEK293 cells expressing hP2X7R carrying D48C/I331C double cysteine mutations. (**a**) Representative currents induced by 4-s application of 300 μM BzATP every 2 min before, during and after exposure to 10 mM DTT in cells expressing WT, D48C, I331C or D48C/I331C mutant hP2X7R. (**b**) Mean BzATP-induced currents at the end of 10-min exposure to DTT (grey) as % of control currents (black). DTT reversibly increased BzATP-induced currents in cells expressing D48C/I331C mutant hP2X7R, but WT, D48C or I331C mutant hP2X7R. Such results are interpreted to indicate that D48 and I331 residues, located in the outer ends of the first and second transmembrane segments, respectively, are in close vicinity in close state, and these parts undergo substantial conformational change during P2X7R activation. Modified from ***(21)***.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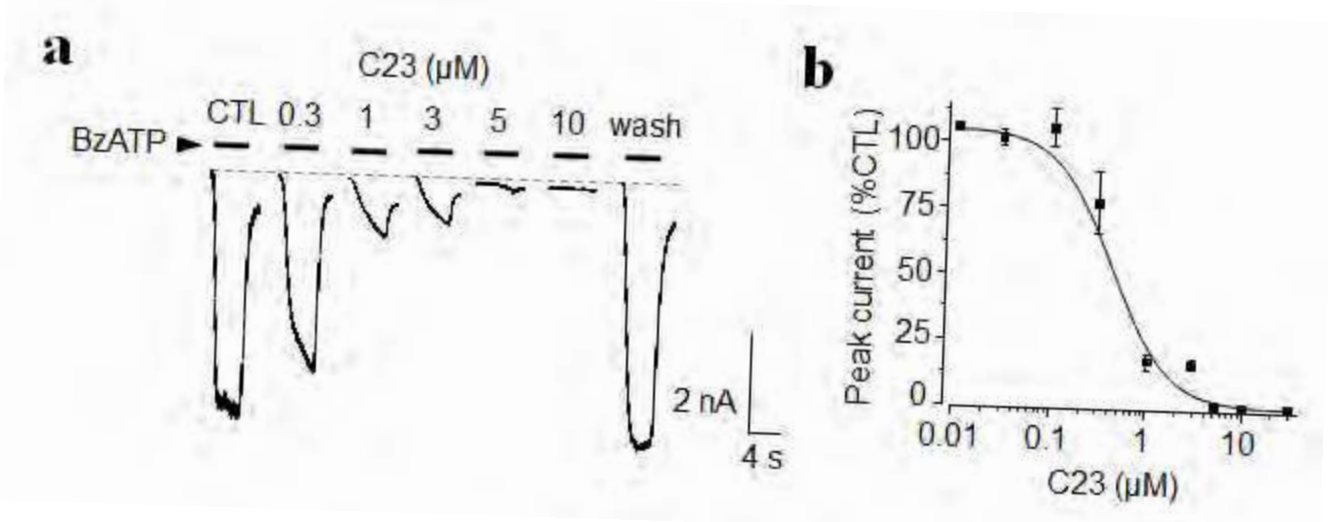
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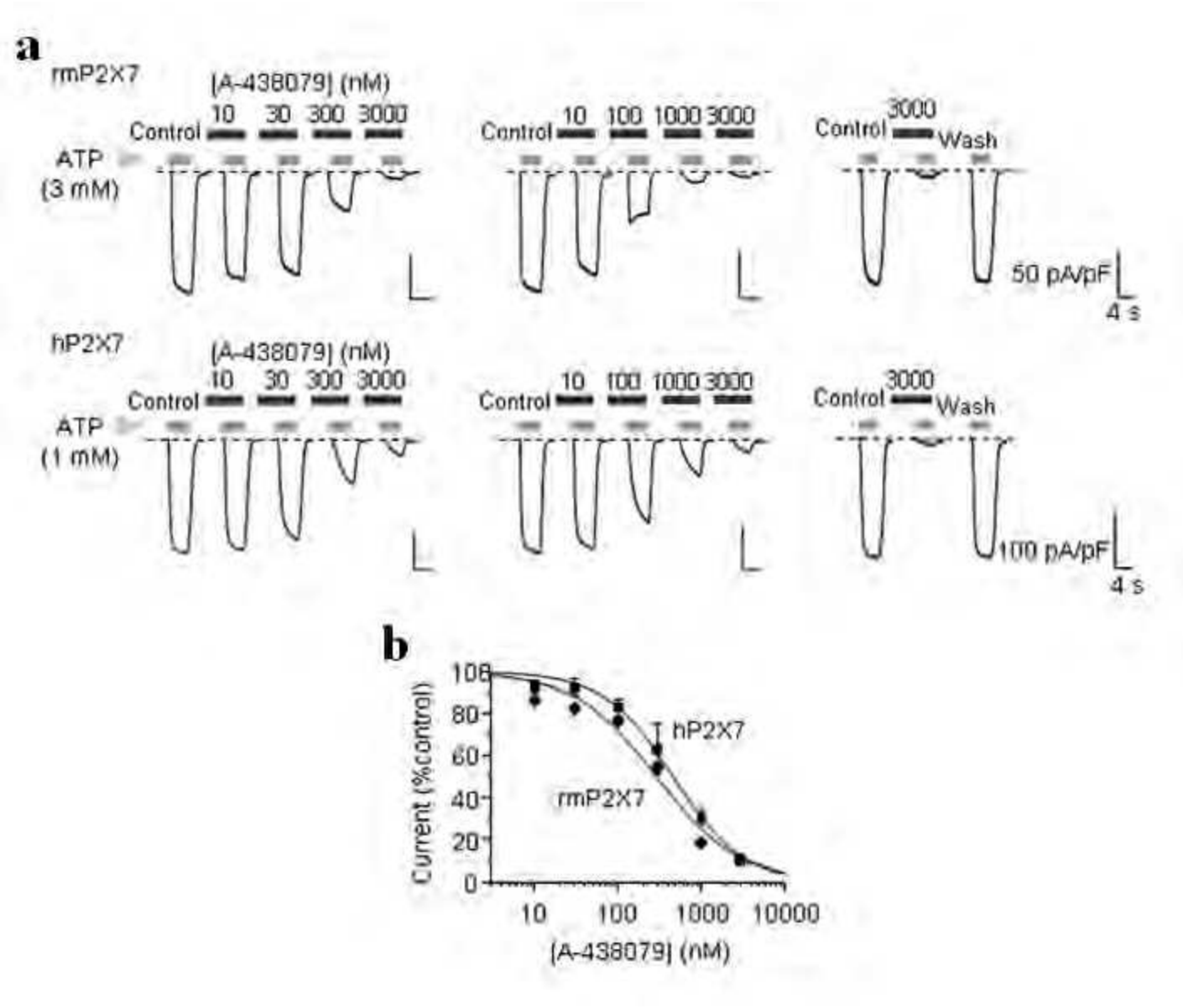
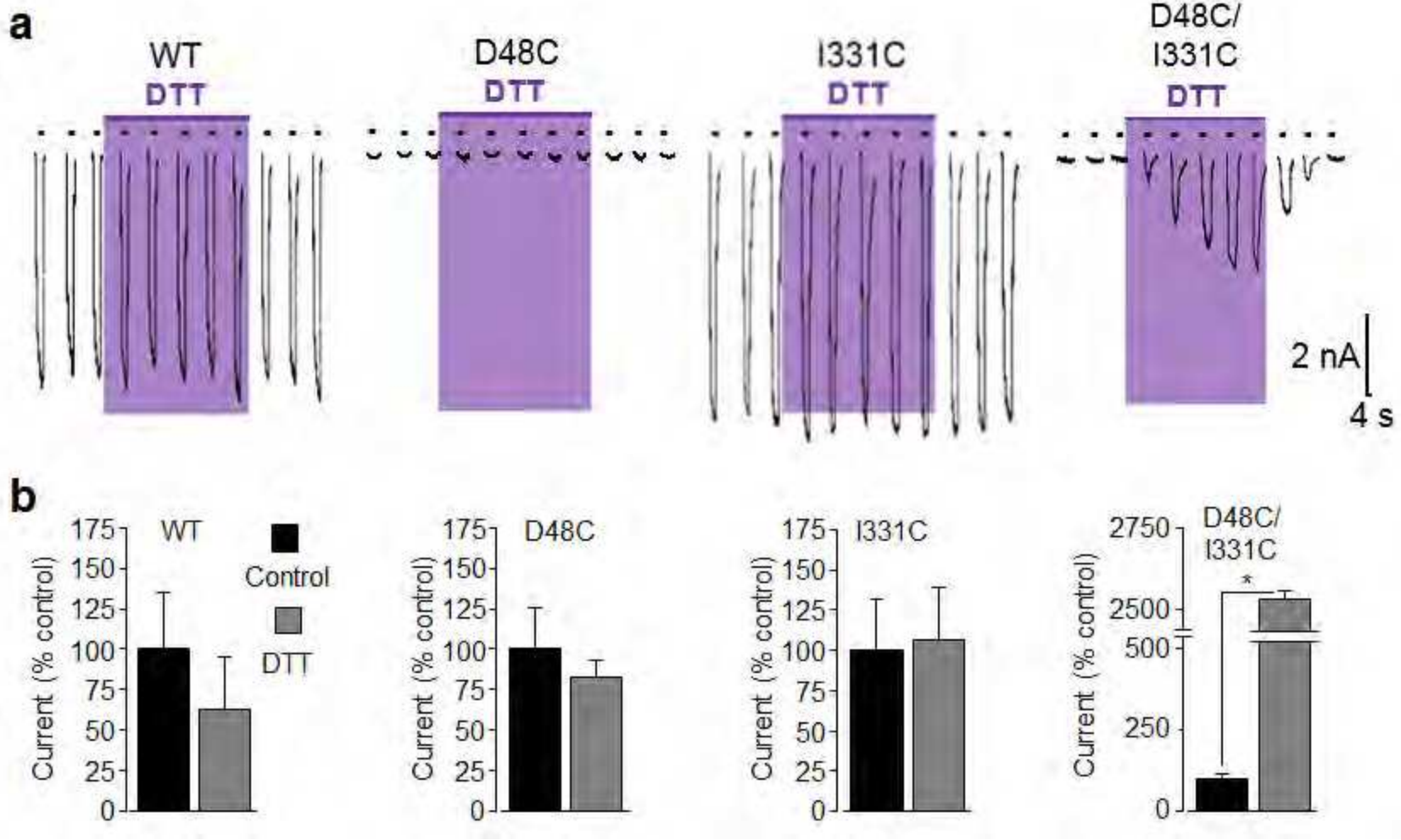
0.1
1
10
C23 (μM)


a
rmP2X7
[A-438079] (nM)
Control
10
30
300
3000
ATP
(3 mM)
Control
10
100
1000
3000
Control
3000
Wash
50 pA/pF
4 s
hP2X7
[A-438079] (nM)
Control
10
30
300
3000
ATP
(1 mM)
Control
10
100
1000
3000
Control
3000
Wash
100 pA/pF
4 s
b
Current (%control)
100
80
60
40
20
0
hP2X7
rmP2X7
10
100
1000
10000
[A-438079] (nM)


a
WT
DTT
D48C
DTT
I331C
DTT
D48C/
I331C
DTT
2 nA
4 s
b
WT
Control
DTT
Current (% control)
175
150
125
100
75
50
25
0
D48C
I331C
D48C/
I331C
2750
2500
500
250
*