PROBING PORE CONSTRICTION IN A LIGAND-GATED ION CHANNEL BY TRAPPING A METAL ION IN THE PORE UPON AGONIST DISSOCIATION

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Supplemental Data

Supplemental Experimental Procedures

Chimeras and mutants. All chimeras used in this study (α7-5HT$_{3A}$R, H–5', H–2', G–5', G–2' and H–2'$^{(RRR\rightarrow QDA)}$) were prepared as described previously (1).

Transfection of cultured cells and cell harvesting. For ligand-binding experiments and membrane preparation, human embryonic kidney (HEK-293T) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine and antibiotics, and grown in 5% CO_2 at 37°C under 90-95% humidity. Cells were transfected with expression vectors (pMT3) carrying cDNA inserts encoding the studied receptors, by using the calcium phosphate precipitation technique (2,3). 10-20 μg DNA was used per 100-mm plate. Two to three days after transfection, when the cells started to lose their normal morphology and became rounded, they were harvested with phosphate buffered saline (PBS) containing 5 mM EDTA and protease inhibitor cocktail (30 μl/100 ml solution; Sigma-Aldrich, Israel). Cells were then centrifuged at 400-800 g for 10 min and the pellet was gently suspended in ice-cold Hepes solution (10 mM Hepes, 140 mM NaCl, 2 mM KCl, 10 mM Glucose, 3.6 mM NaOH, pH 7.4) supplemented with protease inhibitor cocktail devoid of metal-ion chelators. After repeating the last step twice more, the cell pellet was re-suspended in Hepes solution at a ratio of 1 ml per each original cell plate. Ligand-binding experiments were performed within five days from the harvesting date (included), during which cells were kept at 4ºC. Alternatively, harvested cells were subjected to membrane preparation, as follows.

Membrane preparation. Total membranes were prepared on the day of cell harvesting as previously described (4) with slight modifications. Cells (prepared as above) were homogenized with glass-glass or teflon-glass homogenizer in ice-cold Hepes solution supplemented with protease inhibitor cocktail (devoid of metal-ion chelators). The homogenate was centrifuged for 10 min at 800 g in order to remove nuclei and large debris. Supernatant was collected and centrifuged at 18,000 g for 40 minutes. After discarding the supernatant, the pellet was re-homogenized and centrifuged again for 40 minutes at 18,000 g. The latter step was repeated twice more and the pellet was finally suspended in a 0.5-1 ml Hepes solution per each original cell plate. Ligand-binding experiments were performed within five days from the day of membrane preparation (included), during which the membranes were kept at 4ºC.

Ligand-binding assays. To determine the dissociation constant of α-Bungarotoxin (αBTx) binding to the various chimeras, increasing concentrations of ^{3}H-αBTx (40-80 Ci/mmol) (Amersham Life Science, UK)

were added to harvested HEK-293T cells expressing the various receptors. After reaching equilibrium (at least 1 hour on ice), the bound radio-labeled toxin was separated from the unbound radio-labeled toxin by adding 4 ml ice-cold PBS to the tubes containing the binding reaction and pouring the cells onto GFC glass filters (Whatman Inc., USA), which were placed under vacuum. The tube and the filter were further washed twice. Note that the GFC glass filters were pre-incubated in 5% BSA/PBS solution (for at least 30 min) to minimize non-specific adsorption of ^{3}H-αBTx to the filters. Filters were transferred to dry tubes and 4 ml scintillation liquid (Opti-Fluor, PerkinElmer, USA) was added. After 1 hour, the tubes were analyzed in a β counter (Packard Canberra Company, USA). Nonspecific binding was determined as above but in the presence of 2 mM nicotine (Sigma-Aldrich, Israel).

To determine the capacity of the agonists (ACh or nicotine) to inhibit αBTx binding to the various chimeras, cells or membrane preparations were first incubated with increasing concentrations of the agonist for 15 minutes (to reach equilibrium). Then, Zn^{2+} or buffer devoid of Zn^{2+} was added for further 15-min incubation followed by the addition of ^{3}H-αBTx at its K_D concentration for additional incubation of one hour. Assays were performed on ice and were terminated and analyzed as described above. To prevent hydrolysis of ACh, eserine was added (100 μM) before the addition of ACh, while membrane preparations were used to minimize esterase activity typical of cell preparations.

Electrophysiology in Xenopus Oocytes. Stage V and VI *Xenopus* oocytes were prepared as described previously (5). Expression of receptor constructs was obtained by injecting 10 nl of cDNA vector (1 ng) directly into *Xenopus* oocyte nuclei. Two-electrode voltage-clamp measurements were performed at 22-24°C, 2-5 days after DNA microinjection, as described (5). Oocytes were placed into a 100-μl recording chamber (application bath) and were perfused with ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.5 supplemented with 2 mM $CaCl_2$). The perfusion system operated under controlled N2 pressure to enable highly reproducible and constant perfusion velocity of 3.5-3.9 ml/min. The exchange of solutions was performed by electronic valves (ALA-VM8, ALA Scientific Instruments), which are controlled by the computer to allow instantaneous solution exchange. A homemade manifold having only a few microliters of mixing volume together with very narrow connecting tubes prevented backflow of solutions when pressure is electronically switched to another tubing. Taken together, the liquid in the application bath was completely replaced within 1.5-1.7 seconds. Whole-cell currents were recorded by using a GeneClamp 500 amplifier (Axon Instruments). Data acquisition was performed as previously described (5,1).

Supplemental Figure Legends

Supplemental Figure S1. Secondary structure elements of the chimeric H–5' receptor model. Secondary structure elements that correspond to the N-terminal segment are shown in reddish color above the sequence (α, α-helix; β, β-strand, η, 3_{10}-helix; unlabeled thin line, loop structure). Upon subunit assembly, this segment makes up the extracellular ligand-binding domain of chimera H–5' (Fig. 1). It is aligned here with

the template used for its modeling, the acetylcholine-binding protein (AChBP) from *Lymnaea stagnalis* (PDB ID 1UV6) (6). Note that the sequence of the N-terminal segment (amino acids 1-206) corresponds to residues belonging to the chick α7-nicotinic acetylcholine receptor (Swiss-Prot accession no. P22770), except of Ile^{202}-Ile^{203} that were adopted from a mouse serotonin receptor. Black background indicates identical amino acids. Grey background indicates similarities according to size, hydrophobicity, aromaticity, polarity or charge. The secondary structure elements that form the transmembrane channel domain upon receptor assembly are shown in grey, as four transmembrane helices (M1-M4) connected by loops. The sequence of these elements corresponds to residues of the aforementioned serotonin receptor (Swiss-Prot accession no. P23979), except of amino acids Leu^{235}, His^{236} (position −5'), Thr^{238}-Gly^{240} and Thr^{254} (position 13') that were adopted from the β subunit of a glutamate-gated chloride channel from *C. elegans* (Swiss-Prot accession no. Q17328) (see sequence alignment in Fig. 1*C*). Most of the M3-M4 connecting segment is missing (111 amino acids as indicated) due to the lack of a structural template for this sequence.

Supplemental Figure S2. Effects of Zn^{2+} on ACh-activated and spontaneously opened His-containing channels and their Gly-containing control channels. *A-D*, Effects of Zn^{2+} applied externally to *Xenopus* oocytes expressing the chimeric receptors indicated in the panels, after reaching steady-state activation. For activation, 300 μM ACh (a saturating concentration) was used. The increasing concentrations of Zn^{2+} (in μM) are shown within the panels. The current traces shown in panels A-D were adopted from Paas et al (1). *E-H*, ACh-elicited currents (*black traces*) in *Xenopus* oocytes expressing the indicated chimeras. Since these currents are elicited by a saturating concentration of ACh (300 μM), they correspond to the entire activatable population that effectively reflects the resting-state population when the activator (ACh) is absent. The red current traces correspond to the inhibition of the leak currents by externally added Zn^{2+}. Since this inhibition is almost not seen in the glycine-containing controls (panels G & H), the Zn^{2+}-blockable leak currents represent spontaneously opened H−5' and H−2' channels. The equilibrium constant L (used in equation 4, "Experimental Procedures") is defined as the $RESTING_0/ACTIVE_0$ receptor ratio in the absence of the activator. Thus, $L = I_{max(ACh)}/I_{SO}$, where $I_{max(ACh)}$ is the maximal ACh-elicited amplitude (denoted by *'b'*) and I_{SO} is the Zn^{2+}-blockable leak current which is attributed to spontaneously open channels that are blocked by zinc (denoted by '*a'*). For H−5', L = 18 ± 1.5 (mean ± s.e.m., N=6). For H−2', L = 28 ± 3 (mean ± s.e.m., N=7). The resulting L values have been used together with equation 4 ("Experimental Procedures") to simulate the dashed curves in Fig. 3, *D* and *E*. ACh and Zn^{2+} were applied for 6 sec.

Supplemental Figure S3. Induction of receptor deactivation in the presence of Zn^{2+} ions. *A*, Three representative current episodes recorded from *Xenopus* oocytes expressing chimera H−2' are lined up vertically. The first row corresponds to activation by a saturating ACh concentration (300 μM) for 9 sec, which shows the maximal non-desensitizing current amplitude. The second and third rows show application of an ACh-containing solution (300 μM) for 3 sec, which was replaced by a solution containing both ACh (300 μM) and Zn^{2+} (1 mM) for further 6 sec. Then, the ACh + Zn^{2+} solution was replaced by a solution

containing 1 mM Zn^{2+} without ACh, for further 6-sec-long application in order to remove ACh while leaving Zn^{2+} in the application bath. The oocyte was then washed with a physiological solution devoid of ACh and Zn^{2+} for 6 or 12 seconds (*under 1st "wash"*). Then, ACh (300 μM) was reapplied for 3 sec (*under 2nd "ACh"*), followed by a second wash. Note that after each 44-sec-long episode, the oocyte was briefly stimulated for a few times by ACh alone (and washed) to ensure complete recovery of the maximal (primary) amplitude. Recordings were performed at −80 mV, RT. Dashed grey line shows the baseline. Activation time constants are: $\tau = 79 \pm 19$ ms for the first activation and $\tau = 88 \pm 22$ ms for the reactivation (values are mean ± s.d.; N = 5) ($P = 0.51$). *B*, Same as in panel A, but with a control chimera containing a glycine residue instead of a histidine residue at position −2' (G−2').

Supplemental references

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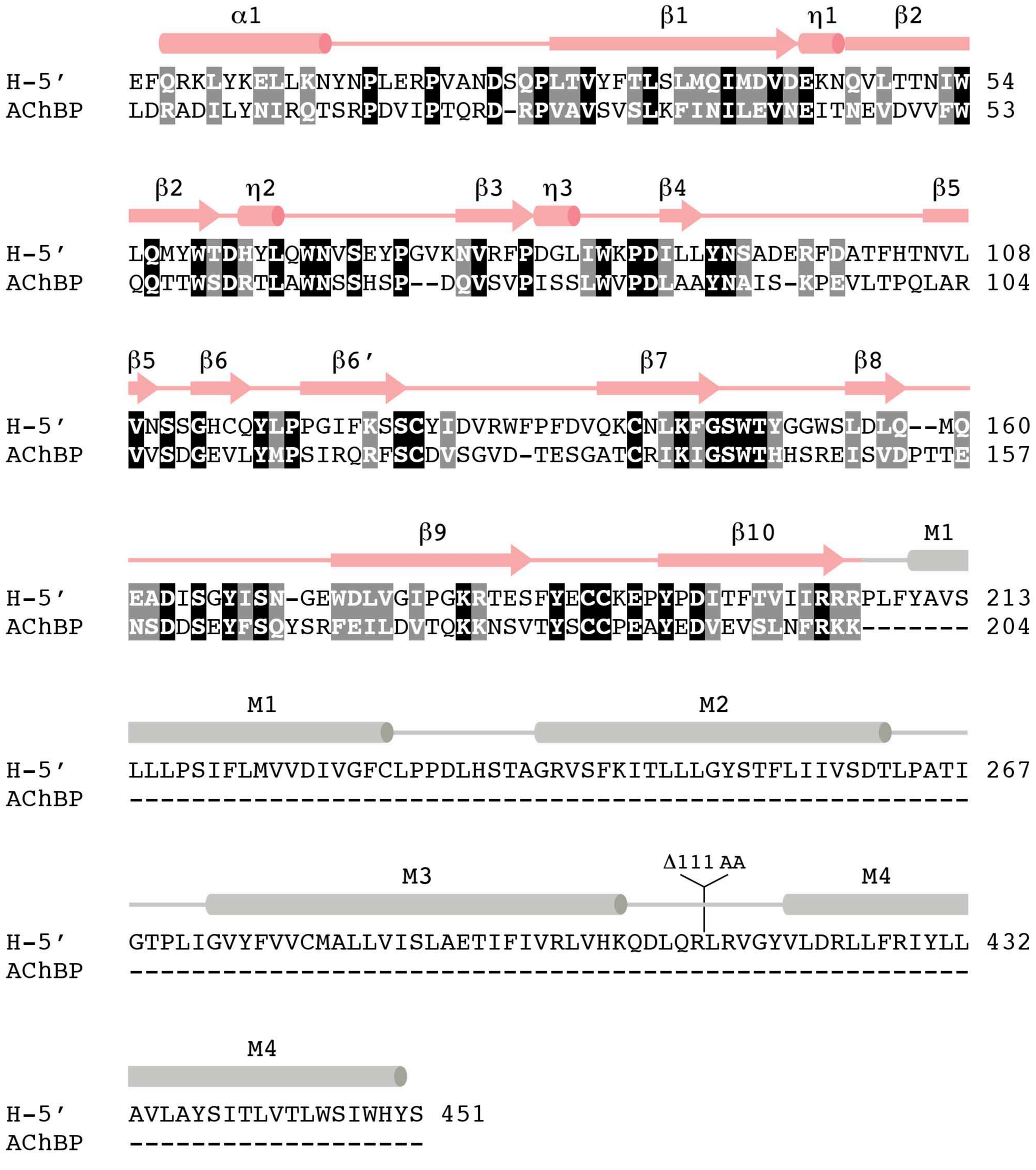

Supplemental Figure S1.
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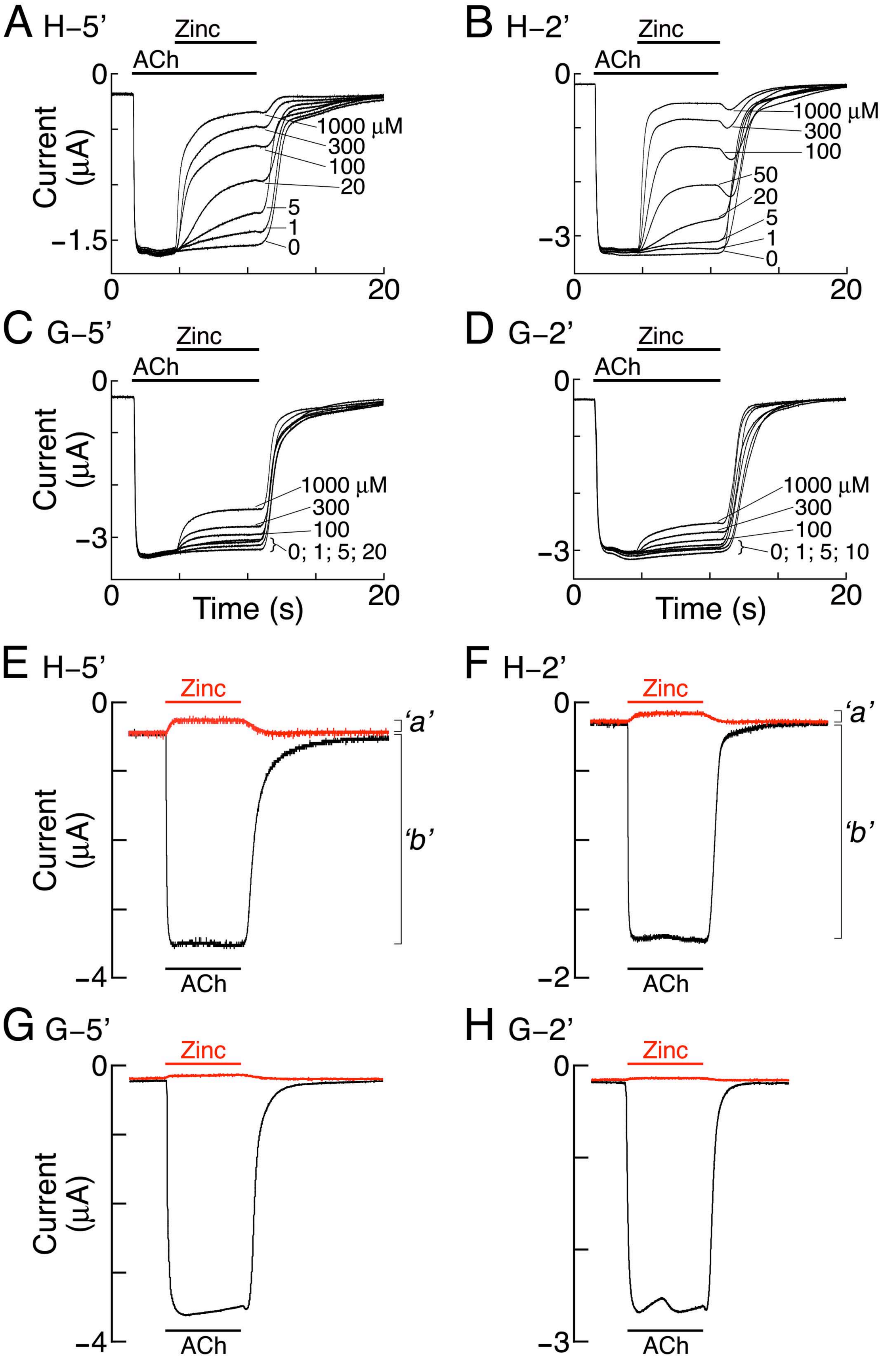
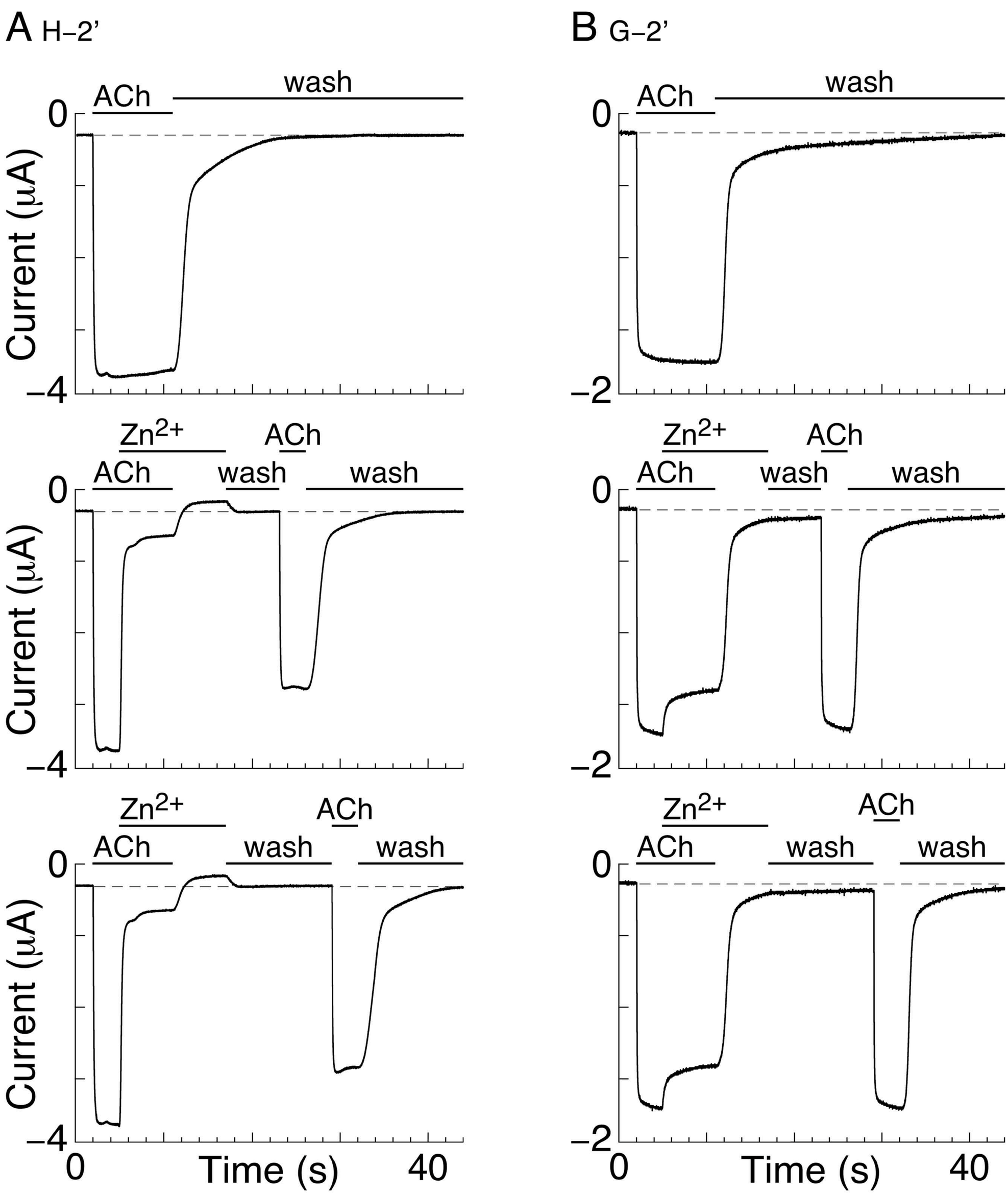


Supplemental Figure S2, Ilya Pittel et al.



Supplemental Figure S3.
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