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Supplementary Information for

Reconstitution of β-adrenergic regulation of $Ca_V1.2$: Rad-dependent and Rad-independent protein kinase A mechanisms.

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SI Methods

Experimental animals and ethical approval

Experiments were approved by Tel Aviv University Institutional Animal Care and Use Committee (IACUC permits # 01-16-104 and 01-20-083). Female frogs were maintained at 20±2°C on 10 h light/14 h dark cycle. Frogs were anaesthetized in 0.2% tricaine methanesulfonate (MS-222), and portions of ovary were removed through an incision on the abdomen. The incision was sutured, and the animal was held in a separate tank until it had fully recovered from the anesthesia, and afterwards was returned to a separate tank for post-operational animals. The animals did not show any signs of post-operational distress and were allowed to recover for at least 3 months until the next surgery. Following the final collection of oocytes (or the 4th surgery as defined by the IACUC rules), anaesthetized frogs were sacrificed by decapitation and double pithing.

DNA constructs and RNA and purified proteins

The cDNA constructs used are: Human Rad (NP_001122322), cardiac long-N terminus isoform of rabbit α_{1C} (GenBank: X15539) and the corresponding mouse α_{1C} isoform (NM_001255999.2), rabbit $Ca_V\beta_{2b}$ (originally termed β_{2a} (1); GenBank: X64297.1; CaVβ2N4 according to the comprehensive nomenclature (2)), α2δ1 (GenBank: M21948). DNA mutations have been introduced using standard PCR-based procedures and verified by sequencing of the coding region of the cDNA. cDNAs of rabbit and mouse α_{1C}Δ821, in which distal C terminus is truncated, were prepared by the removal of the cDNA segment encoding the dCT from the plasmid and introducing a stop codon after valine 1821. Rabbit α_{1C}Δ20Δ1821 was prepared by deletion of the first 20 amino acids of the LNT of α_{1C}Δ1821. The short-NT forms of α_{1C}, with and without the insertion of exon 9*-encoded segment of loop L1, were prepared on the template of rabbit WT α_{1C} (3, 4). Rabbit α_{1C} constructs were in pGEM-GSB or pGEM-HJ vectors containing the 5' and 3' untranslated regions of *Xenopus* β-globin (5), except α_{1C}S1928A that was in pSP72 (6). The latter construct produced small I_{Ba} in oocytes; only cells with I_{Ba}>150 nA were used for analysis. The DNA of the distal C-terminal fragment (dCT) of rabbit α_{1C} encoded a.a. 1821-2171 (8). Two NT mutants of mouse α_{1C} were constructed: α_{1C}NT-4AΔ1821, with alanine substitution of a.a. 2-4; and α_{1C}NT-TYPΔ1821, with alanine substitution of amino acids T_{10}, Y_{13} and P_{15}. Mouse α_{1C} constructs were inserted into pMXT vector, and contained the double mutation T1066Y/Q1070M which renders the channel dihydropyridine-insensitive, but does not affect regulation by PKA (7). The β_{2b}-core construct was prepared by deletion of the first 25 amino acids in the N-terminus of $Ca_V\beta_{2b}$, deletion of amino acids 423-606 in the C-terminus

and removing the linker amino acids 138-202 by PCR (9) (residues numbering according to Opatowsky et al. (10)). β_{2b}-3DA was prepared by introducing 3 point mutations (D244A/D320A/D322A) by PCR using standard site-directed mutagenesis to β_{2b} in which amino acids 423-606 were removed like in β_{2b}-core but the N-terminus and the linker were intact (see Fig. 2A). For technical reasons the C-terminus was extended by adding 17 a.a., GCGSGSGSGSGSGSGPR after a.a. 422. Human CFTR channel (NM_00492) was in pSP64 vector; we note that this cDNA had an extra serine in position 264, which did not affect the channel's function. Human β2 adrenergic receptor (GenBank: AAA88015.1) was subcloned into pGEM-HJ vector. Mouse β1-AR (NP_031445.2) was in pcDNA3.1 vector. For comparison with β2-AR, it was subcloned into pGEM-HJ vector. All other cDNA constructs used for RNA synthesis were inserted into pGEM-GSB or pGEM-HJ vectors, all of which contain 5' and 3' UTR from *Xenopus* β-globin.

The RNAs were prepared using a standard procedure (8). $Ca_V1.2$ was expressed in *Xenopus* oocytes, usually in full subunit composition α_{1C}+α2δ+β_{2b}, by injection of equal amounts, by weight, of RNAs of each subunit (except experiments of Fig. 2 that addressed the role of the $Ca_V\beta$ subunit). We used the long-NT isoform of rabbit α_{1C} (except Fig. S2 where mouse α_{1C} was used) and various mutants, as detailed in the figures. The amount of injected RNA, per oocyte, for full-length α_{1C} was 1.8-5 ng. For α_{1C}Δ1821 constructs, the injected RNA amounts were: 1-1.5 ng for α_{1C}Δ1821, α_{1C}Δ20Δ1821 0.6 ng, α_{1C}NT-4AΔ1821 and α_{1C}NT-TYPΔ1821 3 ng. When $Ca_V\beta$ was not expressed, we injected 5 ng RNA of α_{1C}Δ1821 and $\alpha_2\delta1$. Rad RNA was injected in Rad:β_{2b} ratio of 1:3-1:1. RNA of β1-AR was injected at 5 ng/oocyte if produced on the template of pcDNA vector, or 0.005-0.2 ng when the DNA was cloned into pGEM-HJ. β2-AR was in pGEM-HJ and 0.005-0.2 ng RNA/oocyte were injected. For titration purpose we injected even higher doses (0.5-5 ng), however they decreased the viability of the oocytes and their tolerance to electrode penetration.

His-tagged catalytic-subunit of PKA (His-PKA-CS, GenBank: NM 008854.5) and His-tagged human protein kinase inhibitor protein (PKI) (GenBank: S76965.1) were purified from *E. coli* as described (8), with minor modifications for His-PKA-CS. For details, see SI Methods. PKI was stored and injected into oocytes in PKI buffer (in mM: 20 Tris-HCl, 300 NaCl, 2 DTT, pH 8). His-PKA-CS was stored and injected in PKA buffer (in mM: 20 KH_2PO_4, 20 KCl, 2 DTT, pH 7.5) (8).

Purification of recombinant His-tagged catalytic subunit of PKA

His-tagged catalytic subunit of PKA (His-PKA-CS, GenBank: NM 008854.5) was purified from the *E. coli* strain BL21. Cells were grown in YT medium containing 100 μg/ml ampicillin and 36 μg/ml chloramphenicol at 37° C to an optical density at 600 nm of 0.5-0.8, induced with 1 mM IPTG and grown

for an additional 12h at 16^{o} C, collected by centrifugation, and stored frozen. Cells from 1500 ml of culture were resuspended in 40-ml lysis buffer (50 mM sodium phosphate, 100 mM NaCl, 20 mM Tris-HCl, 5 mM β mercaptoethanol (β-ME), pH 8.0) and protease-inhibitor cocktail tablet (Roche), 2 mM phenylmethylsulfonyl fluoride (PMSF) and lysed in a microfluidizer. Thereafter, cells were centrifuged at 17,000 rpm at 4^{o}C for 30 minutes. The lysate was applied to the Ni-NTA agarose column (2 ml/min). Samples were taken from lysate and flow-through. Then washing was carried out in wash buffer: 50 mM KH_2PO_4, 20 mM Tris–HCl (pH 8), 100 mM NaCl, 10 mM imidazole, 5 mM β-ME. Then elution step was carried out with 40 ml of elution buffer: 250 mM imidazole, 50 mM KH_2PO_4, 20 mM Tris–HCl (pH 8), 100 mM NaCl+ 5 mM β-ME. The eluate was concentrated with Centricon 10 kDa to 5 ml and added to 80 ml gel-filtration buffer (20 mM Mops (pH 8), 100 mM KCl, 5 mM β-ME) and purified on a Superdex G-75 column, subdivided into 4-5 µl aliquots (5µg/µl) and stored at -80^{o}C.

SI Figures

Fig. S1.

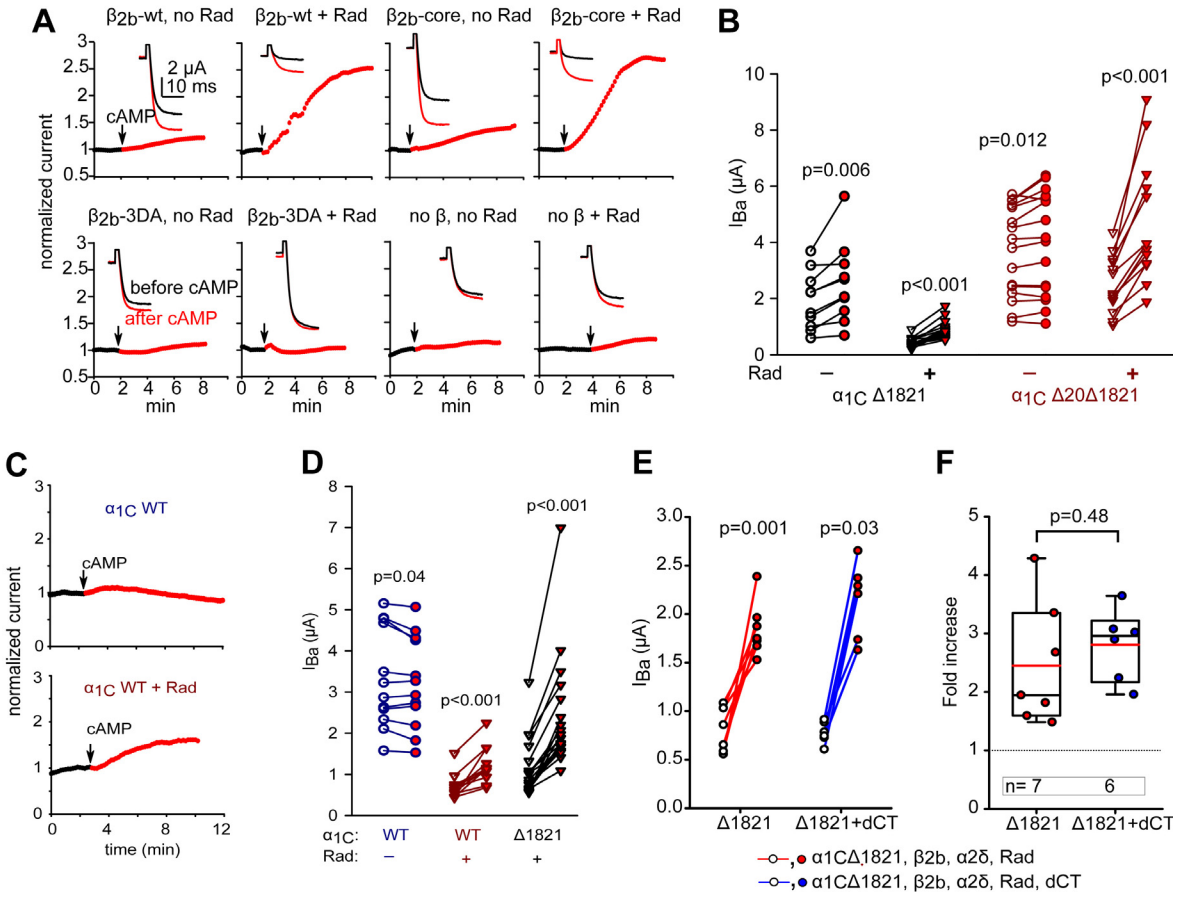

Figure S1. Separation of Rad-dependent and Rad-independent PKA regulation of α_{1C}: the roles of $Ca_V\beta$, N terminus, and the distal C terminus. A, The role of $Ca_V\beta$. A full set of representative diary plots of cAMP-induced changes in I_{Ba} following injection of cAMP (extension of Fig. 2B). Oocytes expressed $\alpha_{1C}\Delta1821$, α2δ, and the indicated variant of β_{2b} (or no β at all), with or without Rad. Insets show current records at +20 mV before (black trace) and 10 min after cAMP injection (red trace). **B,** The role of N-terminal initial segment of α_{1C}. "Before-after" plots of cAMP-induced changes in I_{Ba} in individual cells expressing $Ca_V1.2$ containing $\alpha_{1C}\Delta1821$ (black) and $\alpha_{1C}\Delta20\Delta1821$ (dark red; the latter is lacking the first 20 a.a. of the N-terminus). α2δ and β_{2b} were co-expressed in all cases, without or with Rad (circles and inverted triangles, respectively). N=3 experiments; statistics: paired t-test. For summary of fold increase in these experiments see Fig. 2E. **C,D,** comparison of WT (full-length) α_{1C} and dCT-truncated $\alpha_{1C}\Delta1821$. For summary of fold increase in I_{Ba} in these experiments, see Fig. 2F. **C**, Diary plots of cAMP-induced changes in I_{Ba} in representative cells expressing the full length α_{1C} (α_{1C} WT), α2δ and β_{2b}, without Rad (upper panel) or with Rad (lower panel). **D**, "before-after" plots of cAMP-induced changes in I_{Ba} in individual cells expressing wt-α_{1C} with or without Rad co-expression, or $\alpha_{1C}\Delta1821$ and Rad. α2δ and β_{2b} were present in all cases. Rad:β_{2b} RNA ratio was 1:2 or 1:1. Statistics: paired t-test. N=3 experiments. **E, F,** The clipped distal CT of α_{1C} does not alter Rad-dependent cAMP regulation of $\alpha_{1C}\Delta1821$. **E,** "before-after" plots of cAMP induced changes in I_{Ba} in individual cells co-expressing $Ca_V1.2$-$\alpha_{1C}\Delta1821$ and Rad, with or without the dCT (a.a. 1821-2171) expressed as a separate protein (8). N=1 experiment; statistics: paired t-test (no dCT), Wilcoxon Signed Rank Test (with dCT). **F,** Fold increase in I_{Ba} induced by cAMP injection. 1 experiment; statistics: t-test.

Fig. S2.

Figure S2. Mutations within the initial segment of long-NT disrupt Rad-independent cAMP regulation

A

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rabbit long-NT   MLRALVQPAT PAYQPLPSHL SAETESTCKG TVVHEAQLNH FYISPGGSNY GSPRPAHANM  60
rabbit short-NT  ----MVNENT RMYIPEENHQ ---------- ---------- ------.... ..........  30
rat long-NT      MIRAFAQPST PPYQPLSSCL SEDTERKFKG KVVHEAQLNC FYISPG.... ..........  60
rat short-NT     ----MVNENT RMYVPEENHQ ------ ---------- ----------.... ..........  30
human long-NT    MLRAFVQPGT PAYQPLPSHL SANTEVKFKG TLVHEAQLNY FYISPG.... ..........  60
human short-NT   ----MVNENT RMYIPEENHQ ---------- ---------- ------.... ..........  30
mouse long-NT    MIRAFVQPST PPYQPLSSHS SEETERKFKG KVVHEAQLNC FYISPG.... ..........  60
mouse short-NT   M----VNENT RMYVPEENHQ ---------- ---------- ------.... ..........  30
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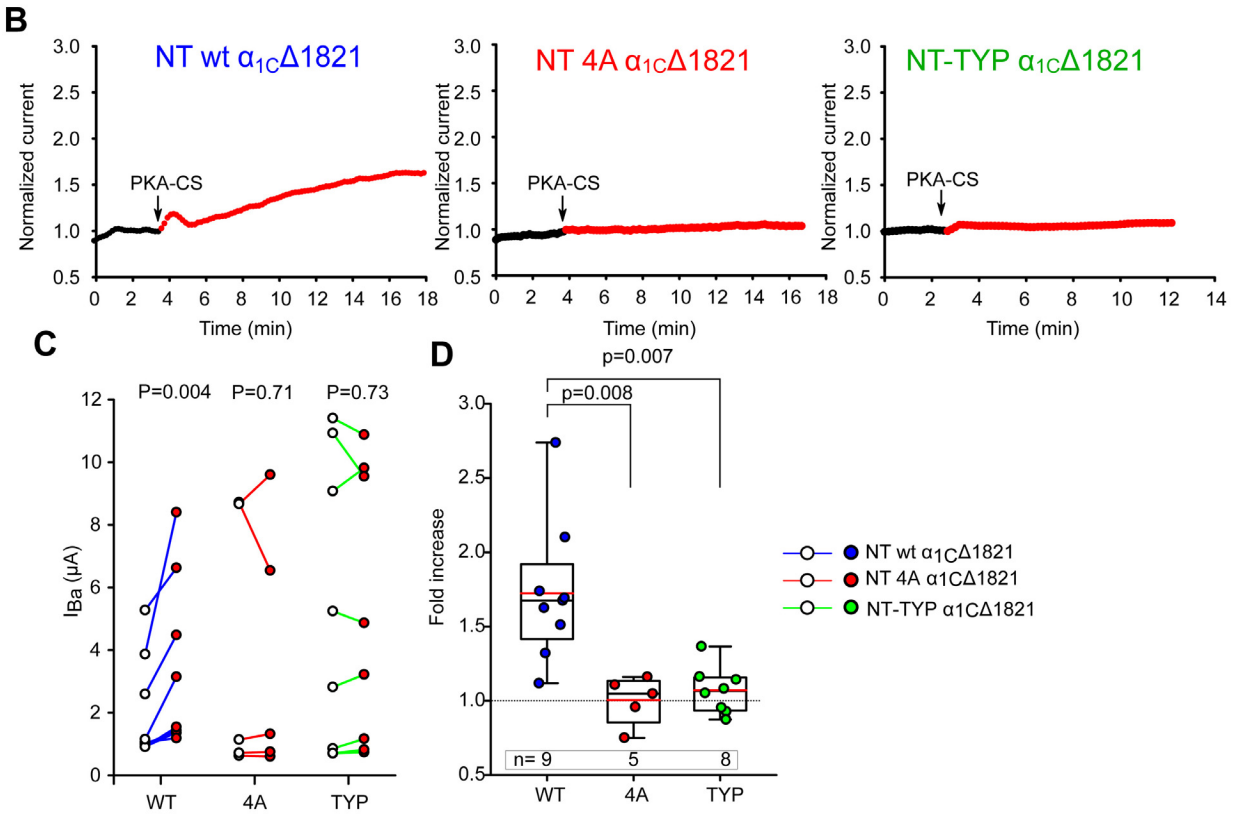

of $Ca_V1.2$-α_{1C}Δ1821. A, Amino acid sequences of the NH_2-terminal part of long-NT and short-NT isoforms of rabbit, rat, human and mouse α_{1C}. Amino acids in positions T_{10}, Y_{13} and P_{15} (TYP motif, marked in red) are conserved in all isoforms. The four a.a. (2-5) of the long-NT isoform mutated to alanine in the NT 4A α_{1C}Δ1821 mutant are highlighted in yellow. The fully conserved a.a. sequence encoded by exon 2 (starting with GSNY) is denoted by dots. **B,** Representative diary plots of normalized Ba^{2+} current at 20 mV. Figure show the time course of change in I_{Ba} following the intracellular injection of 21 ng PKA catalytic subunit (PKA-CS, red trace). The channel was expressed in *Xenopus* oocytes in full subunit composition: α_{1C}Δ1821 (3 ng RNA), β_{2b} (3 ng), $\alpha_2\delta$ (3 ng). In the left panel, the oocytes expressed mouse α_{1C}Δ1821 (NT wt α1CΔ1821). In the middle and right panels, mRNAs of mouse α_{1C}Δ1821 with alanine substitution of amino acids 2-5 (NT 4A α_{1C}Δ1821) and the TYP motif (NT-TYP α_{1C}Δ1821) were used, respectively. **C,** "before-after" plots of PKA-CS induced changes in I_{Ba} in individual cells. N=3 experiments; statistics: paired t-test. **D,** Fold increase in I_{Ba} induced by 21 ng PKA-CS. N=3; statistics: p=0.002, Kruskal-Wallis One Way ANOVA on Ranks followed by Dunnett's test.

Fig. S3.

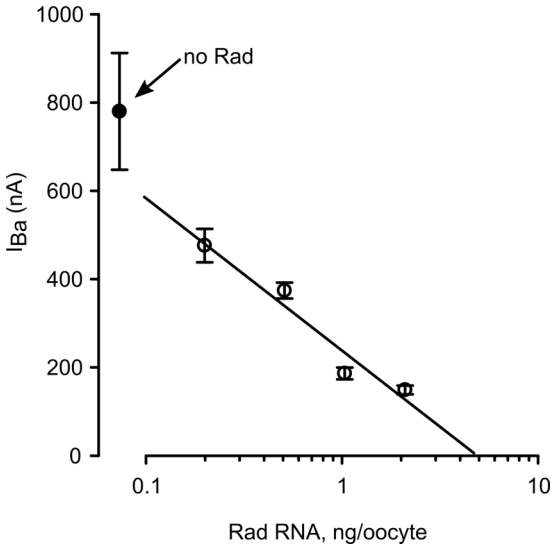

Figure S3. Inverse correlation between Rad concentration and barium currents in β1-AR regulation of $Ca_V1.2$-α_{1C}wt. Rad reduces the Ba^{2+} current of $Ca_V1.2$-α_{1C}wt in a dose-dependent manner. The channel was expressed in full subunit composition: α_{1C}wt (5 ng RNA), β_{2b} (5 ng), $\alpha_2\delta$ (5 ng). I_{Ba} decreased with increasing doses of Rad RNA (Pearson correlation, r=-0.85, p=0.002). Each point presents mean±SEM from 4 to 10 oocytes (N=1 experiment, all oocytes from the same batch and day of recording). The linear regression line was drawn for non-zero doses of Rad.

Fig. S4

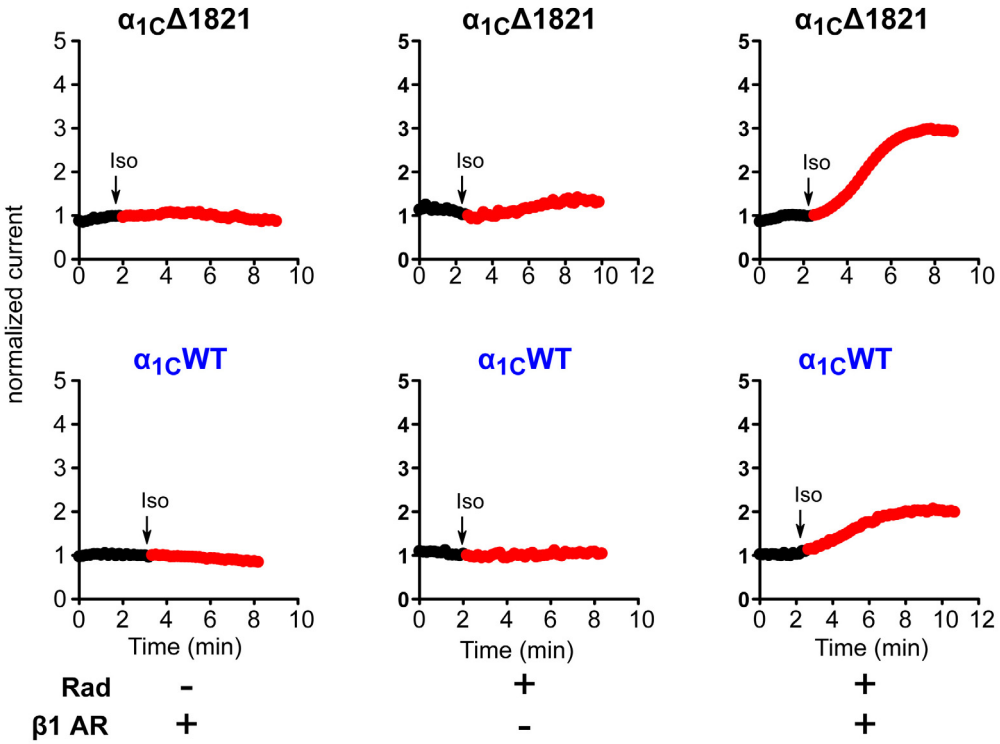

Figure S4. β1-AR regulation of full-length and truncated α_{1C}. Representative diary plots of I_{Ba} in oocytes expressing wt (full-length) α_{1C} ($Ca_V1.2$-α_{1C}) or $Ca_V1.2$-$\alpha_{1C}\Delta1821$ channels (lower and upper panels, respectively) with or without Rad and β1-AR, and the response to 50 μM Iso. β_{2b} and α2δ were coexpressed in all cases. For Summaries, see Fig. 4.

Fig. S5

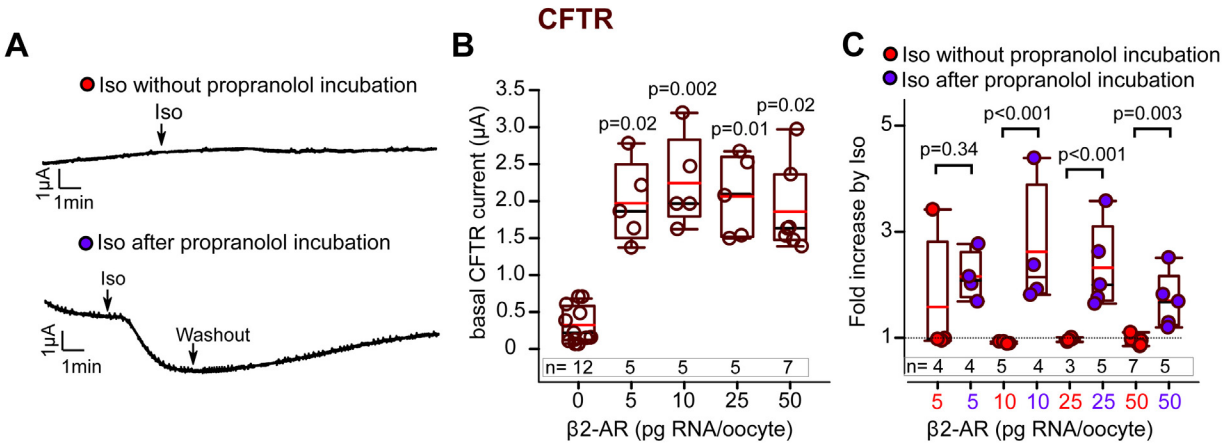

Figure S5. Reconstitution of β2-AR regulation of CFTR. **A,** Representative records in oocytes expressing CFTR and β2-AR. CFTR chloride currents were recorded at -80 mV. Upper panel: without propranolol preincubation; lower panel: with 10 μM propranolol preincubation for 60-120 min. Oocytes were perfused with propranolol-free solution for about 2 min prior to the addition of 50 μm Iso. **B,** Basal chloride currents in oocytes co-expressing CFTR channel (1 ng RNA/oocyte) and β2-AR (increasing doses of RNA, as indicated). Oocytes expressing CFTR alone had significantly lower basal currents than oocytes co-expressing β2-AR (N=1 experiment; Kruskal-Wallis One Way ANOVA on Ranks, H = 22.1, p<0.001). **C,** Fold change in chloride current by Iso. The plot compares the effects of 50 μM Iso in oocytes injected with the indicated doses of β2-AR RNA, without (red symbols) or with (purple symbols) propranolol preincubation. N=1 experiment. Statistics: Mann-Whitney Rank Sum Tests.

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