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

Short title: Reconstitution of β-adrenergic regulation of $Ca_V1.2$

Moshe Katz[1], Suraj Subramaniam[2], Orna Chomsky-Hecht[2], Vladimir Tsemakhovich[1], Anouar Belkacemi[4], Veit Flockerzi[4], Enno Klussmann[5], Joel A. Hirsch[2,3], Sharon Weiss[1], Nathan Dascal[1,3]

[1]Sackler School of Medicine, [2]Faculty of Life Sciences, [3]Sagol School of Neuroscience, Tel Aviv University, Israel; [4]Experimentelle und Klinische Pharmakologie und Toxikologie, Universitat des Saarlandes, 66421, Homburg, Germany; [5]Max Delbrück Center for Molecular Medicine (MDC) in the Helmholtz Association, Berlin, Germany.

Correspondence to: Nathan Dascal, School of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv 6997801, Israel.
tel. (972) 3 6405743; fax. (972) 3 6409113; e-mail: dascaln@tauex.tau.ac.il

Abstract

Introduction

Cardiac L-type voltage-gated $Ca_V1.2$ channels are crucial in physiological regulation of cardiac excitation-contraction coupling. Adrenergic modulation of $Ca_V1.2$ starts with activation of β-adrenergic receptors (AR) and culminates in protein kinase A (PKA) - induced increase of calcium influx through $Ca_V1.2$ channels. To date, this cascade has never been fully reconstituted in heterologous systems; even partial reconstitution proved challenging and controversial. A recent study identified Rad, a calcium channel inhibitory protein, as an essential component of the adrenergic signaling cascade. We corroborated this finding, further characterized, and fully reconstituted, the complete β-AR $Ca_V1.2$ modulation cascade in a heterologous expression system.

Objective

Our primary goal was to heterologously reconstitute the complete β-adrenergic cascade, and to investigate the role of Rad and additional molecular determinants in adrenergic regulation of cardiac $Ca_V1.2$.

Methods and Results: We utilized the *Xenopus* oocyte heterologous expression system. We expressed $Ca_V1.2$ channel subunits, without or with Rad and β1-AR or β2-AR. To activate PKA, we injected cyclic AMP (cAMP) into the oocytes, or extracellularly applied isoproterenol (Iso) to stimulate β-AR. Whole-cell Ba^{2+} currents served as readout. We find and distinguish between two distinct pathways of PKA modulation of $Ca_V1.2$: Rad-dependent (~80% of total) and Rad-independent. We separate the two mechanisms by showing distinct requirements for the cytosolic N- and distal C- termini of α_{1C} and for the $Ca_V\beta$ subunit. Finally, for the first time, we reconstitute the complete pathway using agonist activation of either β1-AR or β2-AR. The reconstituted system reproduces the known features of β-AR regulation in cardiomyocytes, such as a >2-fold increase in $Ca_V1.2$ current, a hyperpolarizing shift in activation curve, and a high constitutive activity of β2-AR.

Conclusions

The adrenergic modulation of $Ca_V1.2$ is composed of two distinct pathways, Rad-independent and Rad-dependent. The latter contributes most of the β-AR-induced enhancement of $Ca_V1.2$

activity, crucially depends on $Ca_V\beta$ subunit, and is differently regulated by β1-AR and β2-AR. The reconstitution of the full β-AR cascade provides the means to address central unresolved issues related to roles of auxiliary proteins in the cascade, $Ca_V1.2$ isoforms, and will help to develop therapies for catecholamine-induced cardiac pathologies.

Introduction

Cardiac excitation-contraction coupling crucially depends on the L-type voltage dependent Ca^{2+} channel, $Ca_V1.2$. Influx of extracellular Ca^{2+} via $Ca_V1.2$ triggers Ca^{2+} release from the sarcoplasmic reticulum, via the Ca^{2+} release channel (ryanodine receptor 2)[1]. Activation of the sympathetic nervous system increases heart rate, relaxation rate and contraction force. The latter is due largely to increased Ca^{2+} influx via $Ca_V1.2$[2]. Pathological prolonged sympathetic activation progressively impairs cardiac function, causing heart failure, partly due to misregulation of $Ca_V1.2$[3].

Cardiac $Ca_V1.2$ is a heterotrimer comprising the pore-forming subunit α_{1C} (~240 kDa), the intracellular $Ca_V\beta_2$ (~68 kDa) and the extracellularly located α2δ (~170 kDa)[4, 5]. The N and C termini (NT, CT respectively) of α_{1C} are cytosolic and vary among $Ca_V1.2$ isoforms (Fig. 1A). It is believed that most of the cardiac α_{1C} protein is post-translationally cleaved at the CT, around amino acid (a.a.) 1800, to produce the truncated ~210 kDa α_{1C} protein and the ~35 kDa cleaved distal CT (dCT); however, the full-length protein is also present[6-9].

The sympathetic nervous system activates cardiac β-adrenergic receptors (β-AR), primarily β1-AR (which mediates most of the βAR-enhancement of contraction and $Ca_V1.2$ activity) and β2-AR[10]. β1-AR is globally distributed in cardiomyocytes, couples to G_s and elevates cAMP levels within the whole cardiomyocyte[10]. In contrast, β2-AR shows spatially restricted localization to the T-tubules (which changes in heart failure)[11] and couples to G_s and $G_{i/o}$[12], producing localized cAMP increases[3].

The cascade of adrenergic modulation of $Ca_V1.2$ comprises agonist binding to β-ARs, activation of G_s, adenylyl cyclase, elevated intracellular cAMP levels, and activation of protein kinase A (PKA). The PKA holoenzyme consists of two regulatory subunits (PKA-RS) bound to two catalytic subunits (PKA-CS). cAMP binds to PKA-RS and causes dissociation of PKA-CS and PKA-

CS. The latter enhances $Ca_V1.2$ activity. However, the final step, how PKA-CS enhances $Ca_V1.2$ activity, remained enigmatic. A long standing paradigm was a direct phosphorylation by PKA-CS of α_{1C} and/or $Ca_V\beta$ subunits[2]. However, numerous studies critically challenged this theory. In particular, mutated $Ca_V1.2$ channels in genetically engineered mice lacking putative PKA phosphorylation sites on α_{1C} and/or β_{2b}, were still upregulated by PKA[7, 13-16] (reviewed in[4, 17]).

One significant obstacle in deciphering the mechanism of PKA regulation of $Ca_V1.2$ was a recurrent failure to reconstitute the regulation in heterologous systems, which proved challenging and controversial[18]. Previous studies in heterologous cellular models, including *Xenopus* oocytes, demonstrated that cAMP failed to up-regulate $Ca_V1.2$ containing the full-length α_{1C}, $Ca_V1.2$-α_{1C}[19-21]. However, robust β-AR – induced upregulation of Ca^{2+} currents was observed in oocytes injected with total heart RNA[22], suggesting the necessity of an auxiliary protein, the "missing link"[19, 20]. Interestingly, partial regulation was observed with dCT-truncated α_{1C}[23, 24], which is considered the predominant form of α_{1C} in the heart[2]. We have previously reported a modest (30-40%) upregulation of $Ca_V1.2$, containing a dCT-truncated α_{1C}, by intracellular injection of cAMP or PKA-CS in *Xenopus* oocytes [24]. Additionally, this regulation required the presence of the initial segment of the long-NT of α_{1C}, but did not involve $Ca_V\beta$ subunit. We proposed that this mechanism may account for part of the adrenergic regulation of $Ca_V1.2$ in the heart[24]. Normally adrenergic stimulation in cardiomyocytes increases the Ca^{2+} current two to three fold, thus a major part of the regulation has remained unexplained.

Recently, Liu et al. identified Rad as the "missing link" in PKA regulation of $Ca_V1.2$[15]. Rad is a member of the Ras-related GTPase subfamily (RGK) that inhibits high voltage activated calcium channels Ca_V1 and Ca_V2[25]. Rad tonically inhibits $Ca_V1.2$, largely via an interaction with $Ca_V\beta$[26, 27]. Ablation of Rad in murine heart was shown to increase basal $Ca_V1.2$ activity but suppressed β-AR regulation[28]. Liu et al. demonstrated that PKA phosphorylation of Rad relieves this tonic inhibition to increase $Ca_V1.2$ activity[15]. They reconstituted a major part of the $Ca_V1.2$ regulation cascade, initiated by forskolin-activated adenylyl cyclase and ultimately attaining a ~2-fold increase in Ca^{2+} current, in mammalian cells expressing α_{1C}, $\alpha2\delta$, β_{2b} and Rad[15]. The relation between this Rad-dependent regulation and the regulation reported in our previous

study[24] is not clear. Furthermore, the complete pathway of adrenergic modulation of $Ca_V1.2$, starting with β-AR activation, has not yet been heterologously reconstituted.

Here we utilized the *Xenopus* oocyte heterologous expression system and, for the first time, reconstituted the entire pathway. We find and distinguish between two distinct pathways of PKA modulation of $Ca_V1.2$: a Rad-dependent and a Rad-independent pathway. We characterize the involvement of the N- and C- termini of α_{1C} and of the β_{2b} subunit in this crucial physiological regulation, and compare and contrast the $Ca_V1.2$ regulation by β1-AR or β2-AR. Our findings reveal novel aspects of the roles of α_{1C} (particularly the N- and C-termini), β_{2b} and Rad in the adrenergic modulation of cardiac $Ca_V1.2$ channels. Reproducing the complete β-AR cascade in a heterologous expression system will promote the identification and characterization of intracellular proteins that regulate the cascade, eventually assisting efforts to develop therapies to treat heart failure and other catecholamine-induced cardiac pathologies.

Methods

Experimental animals and ethical approval

Oocytes were harvested from adult female *Xenopus laevis* frogs, as described[24]. Ethical approval was granted by Tel Aviv University Institutional Animal Care and Use Committee (permits 01-16-104 and 01-20-083).

DNA constructs and RNA

In this work we expressed $Ca_V1.2$ 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. S1 where mouse α_{1C} was used) and various mutants, as detailed in the figures. RNAs of β1-AR, β2-AR, human Rad, or the α_{1C} dCT (as a separate protein) were expressed according to the design of the experiment. The $Ca_V\beta$ subunit used here was rabbit β_{2b} (originally termed β_{2a}[29]). We tested the full-length β_{2b} and β_{2b}-core, composed of a.a. 26-422 of $Ca_V\beta$ with deletion of the linker sequence (a.a. 138-202) as previously described[30]. The β_{2b}-$core_{3DA}$ mutant[31] was prepared based on the β_{2b}-core construct with three point mutations

(D244A/D320A/D322A) (see Fig. 2A). RNAs of all constructs were prepared and injected into oocytes as described[24].

Electrophysiology

Recordings were performed on defolliculated oocytes 3-4 days post RNA injection. Whole-cell Ba^{2+} currents via $Ca_V1.2$ (I_{Ba}) were recorded using two-electrode voltage clamp, routinely in a 40 mM Ba^{2+} solution (in mM: 40 $Ba(OH)_2$,50 NaOH, 2 KOH, and 5 Hepes, titrated to pH 7.5 with methanesulfonic acid). The membrane potential was -80 mV and I_{Ba} was elicited by steps to +20 mV for 20 ms every 10 s. To obtain the current-voltage (I–V) relationship, currents were elicited by 20 ms pulses starting at -70 mV up to +80 mV in 10 mV increments every 10 s. Currents measured in the presence of 200 µM Cd^{2+} were subtracted from total I_{Ba} (Fig. 5A-C) to yield the net I_{Ba}. I-V curves were fitted using the Boltzmann equation[32]. The parameters obtained for G_{max} and V_{rev} were then used to calculate fractional conductance at each V_m and to construct the conductance-voltage (G-V) curve. CFTR currents were measured at −80 mV in ND96 solution (in mM: 96 NaCl, 2 KCl,1 $MgCl_2$, 1 $CaCl_2$, 5 Hepes, pH 7.6).

cAMP, PKA or Iso were applied after verifying that I_{Ba} is stable for at least 2 min. cAMP or PKA-CS were introduced into oocytes by pressure microinjection to final concentrations within the oocyte of ~100 µM (cAMP) or ~25 ng/oocyte (PKA-CS). Isoprenaline hydrochloride (isoproterenol, Iso) was perfused into the experimental chamber.

Statistical analysis

Results are presented as median and interquartile range (IQR) [Q1-Q3], or as mean ± standard error for normally distributed continuous variables. For comparisons between groups, we used Student's t-test or Mann-Whitney test, as appropriate. The fold change in current caused by a treatment in a single oocyte was calculated as (I_{Ba} after treatment)/(I_{Ba} before treatment). The I_{Ba} amplitudes before and after treatment in a single cell were compared using paired t-test or Wilcoxon test, as appropriate. For multiple group comparisons, we performed a One-Way ANOVA or Kruskal Wallis ANOVA on ranks, as appropriate. A Bonferroni post hoc test was performed for normally distributed data (Shapiro-Wilk test) and Dunnett's post hoc test otherwise. Statistical analysis was performed with SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA).

Results

Rad plays a significant role in PKA regulation of $Ca_V1.2$

PKA regulation of heterologously expressed $Ca_V1.2$ was previously observed only with dCT-truncated, but not full-length α_{1C}[23, 24]. In *Xenopus* oocytes, Ba^{2+} currents (I_{Ba}) via $Ca_V1.2$ containing dCT-truncated α_{1C} ($Ca_V1.2$-$\alpha_{1C}\Delta1821$) were increased by 20-40% following intracellular injection of cAMP or PKA-CS[24]. Therefore, we started the study of Rad's role in regulation of $Ca_v1.2$ using $\alpha_{1C}\Delta1821$.

We expressed $Ca_V1.2$ in full subunit composition, $\alpha_{1C}\Delta1821$, β_{2b} and $\alpha2\delta$, unless indicated otherwise, at a 1:1:1 RNA ratio (by weight), and tested the effect of increasing Rad concentrations by injecting varying amounts of Rad RNA. I_{Ba} was measured 3-5 days after RNA injection, by voltage steps from -80 to +20 mV (see Fig. 1C, inserts, for examples of I_{Ba} recordings). As expected[26, 31], basal I_{Ba} was reduced by expression of Rad, showing an inverse correlation with Rad RNA dose (Fig. 1B). Without Rad coexpression, I_{Ba} was increased by 19%±3 (n=32, p=0.002) following cAMP injection (Fig. 1C-E). Coexpression of Rad dramatically augmented the effect of cAMP (Fig. 1C, E). This effect of Rad became statistically significant at Rad:β_{2b} RNA ratios above 1:10, with a maximum increase of 127±10% at 1:2 ratio (Fig. 1F).

The role of $Ca_V\beta$ in Rad dependent regulation

Rad inhibits $Ca_V1.2$ through $Ca_V\beta$-dependent and $Ca_V\beta$-independent mechanisms[26, 31]. To test the role of $Ca_V\beta$ in PKA regulation of $Ca_V1.2$, we compared the change in I_{Ba} following injection of cAMP into oocytes co-expressing $\alpha_{1C}\Delta1821$, $\alpha2\delta$, and β_{2b}-wt (wild-type), β_{2b}-core[30], β_{2b}-$core_{3DA}$ or no β_{2b}, with or without Rad (Fig. 2A). β_{2b}-$core_{3DA}$ was designed on the basis of β_{2b}-core and contained the triple mutation D244A/D320A/D322A which abolishes $Ca_V\beta$-Rad association[31, 35].

In oocytes that did not express Rad, cAMP induced the typical, mild but statistically significant, 20-30% increase in I_{Ba} (Fig. 2B-D). This regulation is termed hereafter “Rad-independent”. The Rad-independent cAMP effect was similar in oocytes that did not express any $Ca_V\beta$ or expressed β_{2b}, β_{2b}-core, or the β_{2b}-$core_{3DA}$ mutant (Fig. 2B-D). Co-expression of Rad

resulted in a much larger, ~2.5-fold increase in peak currents, but only when β_{2b}-wt or β_{2b}-core were present (Fig. 2B, 2D). We refer to this regulation as "Rad-dependent".

Importantly, in Rad-expressing oocytes that did not express $Ca_V\beta$, or expressed the β_{2b}-$core_{3DA}$ mutant, cAMP caused only a mild increase in I_{Ba} that resembled the Rad-independent cAMP effect (Fig. 2B-D). Notably, basal I_{Ba} was significantly increased by coexpression of β_{2b}-$core_{3DA}$ (e.g. Fig. 2C) indicating robust protein expression and channel regulation. Evidently, abrogation of β_{2b}-Rad interaction eliminates the Rad-dependent PKA regulation of $Ca_V1.2$. Thus, Rad-dependent PKA regulation of $Ca_V1.2$ is $Ca_V\beta$-dependent. β_{2b}-core is sufficient to mediate the regulation. In contrast, the Rad-independent mechanism does not require $Ca_V\beta$ (as shown before[24]) and its contribution to cAMP-induced current increase is much smaller than that of the Rad-dependent one.

The N-terminus of α_{1C} is important for Rad-independent but not Rad-dependent regulation

We then set out to characterize the determinants of Rad-independent regulation. The predominant cardiac isoform is the long-NT α_{1C}, where the first 46 a.a., out of ~154, are encoded by the alternative exon 1a[34]. The initial segment (first 20 a.a.) of this α_{1C} isoform acts as an inhibitory module, tonically reducing the activity of $Ca_V1.2$ by reducing the channel's open probability[32]. Within the NT initial segment, a.a. 6-20 show partial homology with the first 16 a.a. of the short NT isoform (these 16 a.a. are encoded by the alternative exon 1), found in smooth muscle and brain[4] (Fig. S1A). Specifically, a.a. T_{10}, Y_{13} and P_{15} (TYP motif) are highly conserved and crucial for the inhibitory function of the NT module[32]. Importantly, the Rad-independent PKA regulation of $Ca_V1.2$-$\alpha_{1C}\Delta1821$ is greatly reduced or abolished by the removal of the first 5 or 20 a.a. of the long NT[24]. We have further addressed the role of the NT initial segment by expressing $Ca_V1.2$ with mouse $\alpha_{1C}\Delta1821$ containing alanine substitutions of a.a. 2-5 (α_{1C}NT-4AΔ1821) or the TYP motif (α_{1C}NT-TYPΔ1821) (Fig. S1, B-D). PKA-CS injection into oocytes expressing $\alpha_{1C}\Delta1821$ with intact NT increased I_{Ba} by ~70% (p=0.004). In contrast, oocytes expressing α_{1C}NT-4AΔ1821 or α_{1C}NT-TYPΔ1821 did not respond to PKA-CS. Thus, as shown previously[24], the initial segment of the long-NT-α_{1C}, including the first 5 a.a. and the TYP motif, is essential for the Rad-independent PKA regulation of cardiac $Ca_V1.2$.

To further address the role of the NT of $\alpha_{1C}\Delta1821$ in Rad-independent vs. Rad-dependent PKA regulation of $Ca_V1.2$, we used NT-truncated-$\alpha_{1C}\Delta1821$ with deletion of the first 20 a.a. ($\alpha_{1C}\Delta20\Delta1821$) (see Fig. 1A). We verified that the Rad-independent increase in I_{Ba} of $\alpha_{1C}\Delta1821$ (34.4±7.5%; n=10) was greatly diminished in $\alpha_{1C}\Delta20\Delta1821$ (5±2%, n=16; $p<0.001$ compared to $\alpha_{1C}\Delta1821$) (Fig. 3A, B). In contrast, co-expression of Rad resulted in similar ~1.7-2 fold increases in I_{Ba} following cAMP injection when $Ca_V1.2$ contained either $\alpha_{1C}\Delta1821$ or $\alpha_{1C}\Delta20\Delta1821$ ($p=0.154$). In both constructs, the fold increase in I_{Ba} was statistically significant in the presence of Rad, and significantly greater than without Rad (Fig. 3). Thus, Rad-dependent regulation does not require the presence of the NT inhibitory module.

The role of distal C-terminus of α_{1C} in PKA regulation

It has been proposed that the cleaved dCT is a potent autoinhibitory domain that reassociates with the truncated α_{1C}, forming a tight molecular complex[8] that is essential for PKA regulation of $Ca_V1.2$[2, 23]. In addition, the cleaved dCT has been reported to traffic to the nucleus where it serves as a transcription regulator[36, 37]. However, in *Xenopus* oocytes, the presence of dCT as a separate protein was not required for Rad-independent regulation of $Ca_V1.2$-$\Delta1821$[24]. Recently, forskolin-induced upregulation of $Ca_V1.2$ was demonstrated in HEK293T cells co-expressing Rad and full-length $Ca_V1.2$ channels[15]. All in all, the role of dCT and its truncation in Rad-dependent regulation remains incompletely understood, and it is unknown whether this PKA regulation equally affects full-length and truncated forms of α_{1C}.

To examine the role of dCT in Rad-dependent PKA regulation of $Ca_V1.2$, we compared the effect of cAMP on either full length (wt) α_{1C} or $\alpha_{1C}\Delta1821$ channels, in the absence and presence of Rad. We also examined the effect of dCT when coexpressed as a separate protein. I_{Ba} is greatly increased by the truncation of dCT[38]. Therefore, to maintain similar macroscopic currents, we injected different amounts of the channel's subunit RNAs: 1-1.5ng RNA for $Ca_V1.2$ containing $\alpha_{1C}\Delta1821$, and 1.8-5 ng RNA for $Ca_V1.2$ containing wt-α_{1C}. The dCT:$\alpha_{1C}\Delta1821$ RNA ratio was 5:1 and Rad:β_{2b} RNA ratios were in the range of 1:3 to 1:1 (which yielded similar increase in I_{Ba} when cAMP is injected; see Fig. 1E). As with $\alpha_{1C}\Delta1821$, co-expression of Rad reduced basal currents of $Ca_V1.2$ containing wt-α_{1C}, with median I_{Ba} of 3.05 µA [IQR 2.46-4.73] without Rad and 0.62 µA [IQR 0.47-0.73] with Rad ($p<0.001$; Fig. 4B).

Injecting cAMP into cells expressing wt-α_{1C} did not increase I_{Ba} (actually, a slight reduction of 4±1.5%, n=12, was observed: Fig. 4A, upper panel; Fig. 4B, C). In contrast, when Rad was coexpressed with wt-α_{1C}, cAMP injection resulted in statistically significant increase of 90±21% in I_{Ba} (n=11) (Fig. 4A, lower panel; Fig. 4B, C). Interestingly, in the same experiments, the Rad-dependent cAMP-induced increase in I_{Ba} appeared higher in oocytes co-expressing Rad with $\alpha_{1C}\Delta1821$: 128±23%, n=18. Pairwise comparison of fold increase in I_{Ba} for $Ca_V1.2$ with wt-α_{1C} vs. $\alpha_{1C}\Delta1821$ showed a mildly significant difference, p=0.04 (Mann-Whitney test, median 2.06 [IQR 1.77-2.63] for $\alpha_{1C}\Delta1821$ vs. median 1.54 [IQR 1.49-2.04] for wt-α_{1C}). When dCT was co-expressed as a separate protein with $\alpha_{1C}\Delta1821$, it did not affect regulation by cAMP even in the presence of Rad (p=0.48) (Fig. S2). Thus, unlike Rad-independent regulation, Rad-dependent PKA regulation does not require the cleavage of dCT. Furthermore, the clipped dCT does not appear to play a role in Rad-dependent PKA regulation of $\alpha_{1C}\Delta1821$. However, there appears to be a quantitative difference in the overall cAMP regulation of full-length versus truncated $Ca_V1.2$.

Full reconstitution of the β1 adrenergic receptor regulation of $Ca_V1.2$

Despite more than a 3-decade effort, it has not yet been possible to reconstitute the entire adrenergic regulation of cardiac $Ca_V1.2$ in a heterologous model. Here we report the reconstitution of the full cascade, starting with activation of β1-AR. The initial experiments were conducted with $Ca_V1.2$ containing $\alpha_{1C}\Delta1821$, using a Rad:β_{2b} RNA ratio of 1:2. In the absence of Rad, isoproterenol (Iso; 50 μM), a non-selective β-AR agonist[39], did not produce any significant increase in I_{Ba} (Fig. 5D, E; Fig. 6). However, co-expression of Rad resulted in a significant increase in I_{Ba} following Iso application. Fig. 5A-B shows traces of currents (Fig. 5A) and current-voltage relationship (Fig. 5B) in a representative oocyte. Fig. 5C shows a conductance-voltage curve drawn from 7 oocytes of the same day's experiment. Iso not only increased currents amplitudes, but also caused a ~5 mV hyperpolarization shift in the $V_{1/2}$ for activation, without changing the slope factor (Fig. 5B, lower panel).

In cardiomyocytes, both full-length (wt) and truncated α_{1C} are present, but it is not known if both isoforms are equally regulated by β1-AR. To study β1-AR regulation of $Ca_V1.2$ containing wt-α_{1C}, we first titrated Rad:β_{2b}, in oocytes that coexpressed β1-AR. As for $\alpha_{1C}\Delta1821$

(see Fig. 1B), we found an inverse correlation between Rad concentration and I_{Ba} (r=-0.85, p=0.002; Fig. S3). In addition, compared with $\alpha_{1C}\Delta1821$, lower Rad:β_{2b} RNA ratios were sufficient to yield a significant increase in currents upon perfusion of Iso (Fig. 5D-E, compare with Fig. 1E). In contrast, in the absence of Rad, there was no increase in currents over time during Iso perfusion (Fig. 5D-E; Fig. 6). Based on the results of Rad titration of Fig. 5, we used Rad:β_{2b} RNA ratio of 1:3 to 1:2 thereafter for channels containing wt-α_{1C}.

We next systematically compared the effect of Iso on channels containing either wt-α_{1C} or $\alpha_{1C}\Delta1821$, with or without coexpressed β1-AR and Rad (Fig. 6). Without the coexpression of Rad, activation of β1-AR did not produce any increase in I_{Ba} in either full-length or truncated channel. This result indicates that the Rad-independent pathway is not activated by β1-AR under the conditions used. Moreover, it appears that oocytes do not contain endogenous Rad or similar RGK proteins that are available for the β1-AR – $Ca_V1.2$ cascade. Interestingly, when oocytes expressed Rad without the receptor, Iso caused a small increase in I_{Ba}: ~15% in $\alpha_{1C}\Delta1821$ (which did not reach statistical significance, p=0.08 by paired t-test), and ~33% in wt-α_{1C} (p=0.016) (Fig. 6B, C). These results corroborate a previous report[40] suggesting that endogenous β-AR is present in some oocyte batches.

In oocytes that expressed β1-AR, Rad and $Ca_V1.2$, Iso induced a robust increase in I_{Ba} (Fig. 6A, B; p<0.001 for both α_{1C} forms). However, comparison of Iso-induced increase in the two α_{1C} forms revealed a significantly greater effect on the truncated channel than on the full-length α_{1C} (147% vs. 87% increase in mean I_{Ba}, respectively; median fold increase 2.2 [IQR 1.72-2.91] versus 1.71 [IQR 1.53-2.07], p=0.002; Fig. 6C). These observations are in agreement with the greater effect of cAMP on the truncated channel (Fig. 4) and imply that the dCT does play a role in attenuating the PKA-induced augmentation of $Ca_V1.2$ currents, at least when it is not cleaved from α_{1C}.

Reconstitution of the β2 adrenergic receptor regulation of $Ca_V1.2$

In healthy heart, the distribution of β2-AR is limited to specific parts of the heart (atrium, apex) and mainly to T-tubules within cardiomyocytes, but becomes more widely distributed over the cardiomyocyte surface in failing heart[11, 41, 42]. β2-AR is also the major β-AR

form in the nervous system[9]. We began with titration of β2-AR with $Ca_V1.2$ containing $\alpha_{1C}\Delta1821$. We expressed the receptors by injecting their RNAs at 50 and 200 pg RNA/oocyte. We also injected higher RNA doses, but above 1 ng/oocyte, cells showed low rate of survival during incubation, and surviving oocytes had high leak currents.

As shown before with cAMP injection (Fig. 1), in oocytes expressing $Ca_V1.2$-$\alpha_{1C}\Delta1821$, Rad and β1-AR, a robust regulation of I_{Ba} by Iso was observed (Fig. 7A upper trace, Fig. 7 B, C). Surprisingly, in oocytes of the same batch expressing $Ca_V1.2$-$\alpha_{1C}\Delta1821$, β2-AR and Rad, I_{Ba} did not respond to Iso (Fig. 7A lower panel, Fig. 7B-C). In some cases, we did observe an increase in I_{Ba} (e.g. Fig. 8G) but it was very small compared to β1-induced $Ca_V1.2$ stimulation. To test if the expressed β2-AR was functioning well, we used cystic fibrosis transmembrane conductance regulator (CFTR) channel as a control. PKA phosphorylation of CFTR, a chloride channel, activates the channel, leading to an increased outward chloride current[43]. Moreover, CFTR is robustly activated by cAMP and PKA-CS injection in *Xenopus* oocytes[44].

Co-expressing CFTR with a range of β2-AR doses (RNA range, 5-50 pg/oocyte) was associated with significantly higher basal chloride currents (I_{CFTR}) compared with cells expressing CFTR alone ($p<0.001$) (Fig. 8B). Iso perfusion did not cause an increase in I_{CFTR} at any of the β2-AR doses (Fig. 8A upper panel, Fig. 8C, red circles). This observation implied that CFTR channels may have already been opened by agonist-independent constitutively active β2-ARs, thus blunting any response to Iso. Reducing the basal activity of the receptor would result in lower chloride basal currents and may reinstate the activation of β2-AR upon Iso perfusion. Propranolol is a non-selective β blocker and an inverse agonist, which is known to reduce the constitutive activity of β2-AR[45]. Therefore, we incubated the oocytes for 1-2 hours with 10 µM propranolol before starting the recording. Just before the recording, the oocyte was placed in the experimental chamber, voltage clamp was established, and the cell was washed with propranolol-free solution for 2-4 minutes before application of Iso. With propranolol preincubation, we found that Iso induced a robust 1.5-3 fold increase in chloride currents in oocytes expressing CFTR and β2-AR (Fig. 8A lower panel and Fig. 8C, purple circles).

The results of the CFTR experiment strongly supported the possibility that high constitutive activity of β2-AR precluded further effect of Iso, also in the case of $Ca_V1.2$.

Therefore, we employed the propranolol preincubation protocol for $Ca_V1.2$-$\alpha_{1C}\Delta1821$, coexpressed with β2-AR and Rad. We measured I_{Ba} in oocytes not preincubated (black) or pre-incubated (purple) with propranolol (Fig. 8D). As predicted, preincubation with propranolol resulted in a statistically significant reduction of basal I_{Ba} (Fig. 8D). Application of Iso produced only a mild but significant increase in I_{Ba} without propranolol incubation, by 24±5%, in this experiment (Fig. 8E, G, H) compared to a significantly greater increase, 3-5 fold, with propranolol pre-incubation (Fig. 8F-H). We conclude that we successfully reconstituted adrenergic regulation of the $Ca_V1.2$ channel current as mediated by the two canonical and physiologically-relevant adrenergic receptors.

Discussion

Numerous biological regulations of ion channels that have been fully reconstituted in heterologous systems accelerated the understanding of their mechanisms and structure-function relationships. However, the classical adrenergic regulation of cardiac L-type Ca^{2+} channel remained an unmet challenge for several decades. The recent discovery of the crucial role of Rad[15] was a turning point. Here we report, for the first time, the heterologous reconstitution of the full cascade of β-AR regulation of the cardiac L-type Ca^{2+} channel, $Ca_V1.2$, in *Xenopus* oocytes, starting with the receptor. Two major advantages of the *Xenopus* oocyte model are accurate titration of protein expression (by titrated RNA injection), and the ability to co-express a large number of proteins. We utilized the simplicity and robustness of the oocyte expression system to reconstitute the full β-AR cascade, to address the role of Rad, the relation between Rad-dependent and the previously reported Rad-independent PKA regulation[24], and to elaborate the role of $Ca_V\beta$ and the distal parts of N- and C-termini of α_{1C}.

First, we confirmed the cAMP-induced upregulation of I_{Ba} in oocytes expressing $Ca_V1.2$-$\alpha_{1C}\Delta1821$ without Rad[24] which, in this series of experiments, was ~20%. This is less than the previously reported 30-40%[24], probably because here we injected about half the amount of cAMP. Notably, a greater Rad-independent I_{Ba} potentiation was attained by injecting purified PKA-CS (Fig. S1).

Next, we scrutinized the role of Rad, initially using cAMP injection to activate the oocyte's endogenous PKA. Liu et al. reported a ~1.5-2-fold increase in maximal conductance

(G_{max}) of $Ca_V1.2$ containing the full-length α_{1C} in HEK cells[15]. Here, with $\alpha_{1C}\Delta1821$, titrated expression of Rad showed the expected[26, 27] Rad-dose-dependent decrease in I_{Ba}, paralleled by a robust enhancement in cAMP-induced increase in I_{Ba}, up to ~2.2-fold (Fig. 1). These results confirm the importance of Rad in PKA regulation and, moreover, suggest that Rad-dependent regulation applies to both full-length and C-terminally-truncated α_{1C}. Importantly, the Rad-induced enhancement by cAMP leveled off and occasionally seemed to diminish at higher Rad:$Ca_V1.2$ RNA ratios (we expressed all channel subunits in equal weights doses), suggesting that molar excess of Rad may override the PKA-induced abolition of tonic Rad inhibition. Thus, we used the optimal Rad:β_{2b} RNA ratio, 1:3 – 1:1, in all experiments.

We next inquired whether Rad-dependent and Rad-independent regulatory mechanisms are distinct, considering the possibility that the latter was actually the same as Rad-dependent regulation, possibly mediated by an endogenous RGK protein in the oocyte. Our results unequivocally demonstrate that the two modalities are carried out by distinct molecular mechanisms. First, Rad-dependent regulation was fully $Ca_V\beta$-dependent, as demonstrated[15]. Both Rad inhibition of the basal I_{Ba}, and the Rad-dependent enhancement of cAMP effect critically depended on the presence of coexpressed $Ca_V\beta$ subunit (full-length or core), and both Rad actions were suppressed by a triple mutation that abolishes Rad-$Ca_V\beta$ interaction (Fig. 2). In contrast, the Rad-independent cAMP effect was the same with all β_{2b} constructs used, or without coexpressed $Ca_V\beta$. Second, as shown before[24], the Rad-independent regulation crucially depended on two cytosolic elements of α_{1C}: the inhibitory module (initial segment) of the NT (Fig. 3, Fig. S1), and truncation of dCT of α_{1C} (Fig. 4). In contrast, in the presence of Rad, robust regulation of I_{Ba} by cAMP was consistently observed, both after the deletion of the initial NT segment, and in channels containing either full-length or dCT-truncated α_{1C}.

We then reconstituted the full β-AR cascade with coexpressed β1-AR. Activation of β1-AR by Iso caused a >2-fold increase in $Ca_V1.2$-$\alpha_{1C}\Delta1821$ current and the typical hyperpolarizing shift in voltage dependence of activation (Fig. 5, 6), resembling cardiomyocytes[16]. Coexpression of G_s and adenylyl cyclase was not necessary, suggesting sufficient levels of endogenous proteins. However, expression of Rad was essential; in its absence, no increase in I_{Ba} was observed. It is unclear why the Rad-independent regulation of $Ca_V1.2$-$\alpha_{1C}\Delta1821$ could not be

produced by activation of β1-AR. We consider several possibilities, among them another missing factor needed for this regulation, or a stoichiometry predicament with either the receptor or a downstream protein of the cascade. Unfortunately, expressing high doses or β1-AR, $G\alpha_s$ or adenylyl cyclase consistently resulted in high oocyte mortality. Whatever the reason, it appeared that, under the conditions of our experiments, β1-AR regulated $Ca_V1.2$ only via the Rad-dependent mechanism.

Of particular importance is the observation that, once Rad was present, both dCT-truncated and full length channels were upregulated both by cAMP and by β1-AR (Figs. 5, 6). This was never clear, and previously controversial[18]. The full-length α_{1C} is present in the heart and seems even more abundant in neurons[9]. Yet, the mechanism of regulation of neuronal $Ca_V1.2$ appears different from that of cardiac; the direct PKA phosphorylation of serine 1928 (located in the dCT) is highly important in neurons[46], but not in the heart[13, 47]. However, in our system the β1-AR activation of full-length α_{1C}-based channels (containing S1928) still required Rad. This indicates that phosphorylation of S1928 alone is not enough. Interestingly, a more detailed examination of the results reveals a potentially important difference: with cAMP, and even more so with β1-AR, the overall regulation of the dCT-truncated channel is significantly stronger than of the full-length channel. A more straightforward interpretation would be the contribution of Rad-independent regulation, which is present only in $\alpha_{1C}\Delta1821$. However, this is valid only for cAMP-induced regulation; as discussed, β1-AR does not appear to initiate the Rad-independent pathway. We propose that the dCT exerts a regulatory control over the Rad-dependent β-AR regulation of the channel, via mechanisms that need to be explored.

Finally, we were also able to reconstitute the $Ca_V1.2$ enhancement by β2-AR which, like β1-AR, acted via the Rad-dependent pathway (Figs. 7, 8). However, there was a significant difference. As demonstrated with both CFTR and $Ca_V1.2$, β2-AR constitutively activated the G_s-PKA pathway, rendering very high basal CFTR and $Ca_V1.2$ currents, as revealed by the inverse agonist, propranolol (Fig. 8). The basal $Ca_V1.2$ activation was so strong even with very low doses of β2-AR RNA, that pretreatment with propranolol was required to observe upregulation by Iso. Whereas high basal constitutive activity of β2-AR is well established[45, 48, 49], in the heart, agonist stimulation of β2-AR normally increases $Ca_V1.2$ currents[39, 42]. We assume that, in

cardiomyocytes, specific mechanisms such as restricted localization[9, 41, 42] or additional auxiliary proteins may regulate the basal activity of β2-AR.

In summary, we reconstituted the β1-AR and β2-AR regulation of $Ca_V1.2$ in the *Xenopus* oocyte model system. We demonstrate a robust (~2-fold) enhancement of Ca^{2+} channel currents, by cAMP and Iso, in a Rad-dependent manner, and confirm the existence of a less prominent, separate, Rad-independent PKA regulation of $Ca_V1.2$ (~30% increase in $Ca_V1.2$ current). Our reconstituted system replicates the known basic features of β-AR regulation of cardiac $Ca_V1.2$ and reveals novel molecular details, including the consequences of proteolytic processing of α_{1C} with respect to β-AR regulation, the differential roles of $Ca_V\beta$ subunit and of the N- and C-termini of α_{1C} in the Rad-dependent and Rad-independent regulation, and the differences in channel regulation by β1-AR and β2-AR. The reconstitution of the basic cascade will enable further investigation of the mechanisms of Rad action and additional auxiliary proteins implicated in macromolecular complexes involved in β-AR regulation of $Ca_V1.2$, such as A-kinase anchoring proteins, phosphatases and phosphodiesterases, and others[50]. It may also be instrumental in identifying potential targets for therapeutic modulation of β-adrenergic regulation in heart and other tissues.

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Figure legends

Figure 1.

Figure 1. cAMP regulation of $Ca_V1.2$ is greatly enhanced by co-expression of Rad. A, The cardiac $Ca_V1.2$ and Rad. The α_{1C} and α2δ subunits are shown schematically, along with structures of β_{2b} (PDB:1T3S)[30] and Rad (PDB: 3Q72)[33]. In α_{1C}, the four homologous transmembrane domains are numbered I to IV. The constructs used here were based on rabbit or mouse long-NT isoforms, containing the 46 a.a.-long initial segment encoded by exon 1a[34] and either full-length CT, or, in $\alpha_{1C}\Delta1821$, the α_{1C} was truncated at a.a. 1821, close to the naturally occurring truncation site (red cross mark) in cardiac α_{1C}, ~a.a. 1800[7]. β_{2b} binds to the cytosolic loop connecting domains I and II. When recruited to the plasma membrane, Rad interferes with the interaction between β and α_{1C} subunits. **B-F,** examples and summaries for $Ca_V1,2$-$\alpha_{1C}\Delta1821$. **B**, Rad reduces the Ba^{2+} current of $Ca_V1.2$-$\alpha_{1C}\Delta1821$ in a dose-dependent manner. The channel was expressed in full subunit composition: $\alpha_{1C}\Delta1821$, β_{2b} and α2δ (1.5 ng RNA of each subunit). I_{Ba} decreased with increasing doses of Rad RNA (Pearson correlation, r=-0.82, p=0.023). Each point represents mean±SEM from 7 to 10 oocytes (N=1 experiment). The linear regression line was drawn for non-zero doses of Rad. **C**, Rad enhances the cAMP-induced increase in I_{Ba}. Figures are diary plots showing the time course of change in I_{Ba} (normalized to initial I_{Ba}), before and after the intracellular injection of cAMP in a representative cell. Insets show current records at +20 mV before (black trace) and 10 min after cAMP injection (red trace), without Rad in the upper panel and with Rad in the lower panel. **D**, "before-after" plots of cAMP-induced changes in I_{Ba} in individual cells injected with increasing ratio of Rad:β_{2b} expression. Empty symbols – before cAMP; red-filled – after cAMP. RNA ratios (by weight, w/w) of Rad and β_{2b} were varied in 1:20 to 2:1 range. N=3 experiments; statistics: paired t-test. **E**, cAMP-induced increase in I_{Ba} depends on relative expression levels of Rad and β_{2b}. Each symbol represents fold increase in I_{Ba} induced by cAMP injection in an individual cell, at different Rad:β_{2b} RNA ratios. Here and in the following figures, box plots show 25-75 percentiles, whiskers show the 5/95 percentiles, the black and the red horizontal lines within the boxes are the median and mean, respectively. At all Rad:β_{2b} RNA ratios except 1:20, the cAMP-induced

increase in I_{Ba} was significantly greater than without Rad (Kruskal-Wallis One-Way ANOVA on ranks). **F**, summary of the effect of cAMP in 10 experiments without Rad and with Rad at 1:2 and 1:1 Rad:β_{2b} RNA ratios (pooled). Number of cells is shown within the bars. Statistics: Mann-Whitney test.

Figure 2.

Figure 2. Separation of Rad-dependent and Rad-independent PKA regulation of α_{1C}: the role of $Ca_V\beta$. A, Schematic representation of $Ca_V\beta$ subunit variants used in this study. The wild-type (β_{2b}-wt) protein is 632 a.a. long. β-core is truncated at a.a. 424; the linker amino acids 138-202 were removed[30]. The β-core$_{3DA}$ is the β-core with three aspartate-to-alanine mutations, D244A/D320A/D322A, that does not bind Rad.. **B-D**, the presence of the β subunit and its ability to bind Rad are crucial for Rad-dependent, but not for Rad-independent, cAMP regulation of $Ca_V1.2$. Rad:β_{2b} RNA ratio was 1:1. N=1 experiment. **B**, Diary plots of cAMP-induced changes in I_{Ba} following injection of cAMP in representative cells expressing $\alpha_{1C}\Delta1821$, 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). **C,** "Before-after" plots of cAMP-induced changes in I_{Ba} in individual cells injected with various $Ca_V\beta$ subunits co-expressed with and without Rad. N=1 experiment; statistics: paired t-test. **D**, Fold change in I_{Ba} after cAMP injection. Data show the fold increase with Rad co-expression (inverted triangles) and without Rad (circles). Mann-Whitney Rank Sum test was used to compare groups with and without Rad (except the groups with β-core$_{3DA}$ where normality was satisfied, and t-test was used).

Figure 3.

Figure 3. Separation of Rad-dependent and Rad-independent PKA regulation of α_{1C}: the role of N-terminal initial segment of α_{1C}. A, "before-after" plots of cAMP-induced changes in I_{Ba} in individual cells expressing $Ca_V1.2$ containing $\alpha_{1C}\Delta1821$ (black) and $\alpha_{1C}\,\Delta20\Delta1821$ (red) 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. **B,** Fold change in I_{Ba} after cAMP injection (summary of experiments shown in A). The black and the

red horizontal lines within the boxes are the median and mean, respectively. Pairwise comparison with and without Rad was done using the Mann-Whitney Rank Sum test.

Figure 4.

Figure 4. Separation of Rad-dependent and Rad-independent PKA regulation of α_{1C}: the role of the distal C-terminus of α_{1C}. A, Diary plots of cAMP-induced changes in I_{Ba} in representative cells expressing the full length α_{1C} (α_{1C} WT), $\alpha 2\delta$ and β_{2b}, without Rad (upper panel) or with Rad (lower panel). **B**, "before-after" plots of cAMP-induced changes in I_{Ba} in individual cells expressing wt-α_{1c} with or without Rad co-expression, or $\alpha_{1C}\Delta 1821$ and Rad. $\alpha 2\delta$ 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. n=12, 11 and 18 oocytes, from left to right. **C,** Fold change in I_{Ba} at +20 mV after cAMP injection (summary of data shown in A, B). The black and the red horizontal lines within the boxes are the median and mean, respectively. Statistics: Kruskal-Wallis One-Way ANOVA on ranks.

Figure 5.

Figure 5. Full reconstitution of the β1-AR regulation of α_{1C}. A-C, β1-AR regulation of $Ca_V1.2$. Oocytes were injected with RNA of $\alpha_{1C}\Delta 1821$, $\alpha 2\delta$, β_{2b}, Rad and β1-AR. **A**, Ba^{2+} currents (upper panels) before (left) and after (right) perfusion of 50 µM isoproterenol (Iso) in a representative cell. The voltage protocol is illustrated in the lower panel; I_{Ba} was elicited by 20-ms voltage steps given every 10 s from a holding potential of -80 mV in 10 mV increments. The currents shown are net I_{Ba} derived by subtraction of the residual currents recorded with the same protocols after applying 200 µM Cd^{2+} (not shown). Since full capacity compensation in oocytes was not achievable, the currents during the first ~2 ms (the duration of capacity transient) were blanked out. **B**, Top, representative I-V curve before (black) and after (red) addition of Iso. Bottom, parameters of Boltzmann fit of I-V curves in 7 oocytes, before and after Iso. **C**, Conductance-voltage (G-V) curves of $Ca_V1.2$-$\alpha_{1C}\Delta 1821$ co-expressed with Rad and β1-AR averaged from oocytes of a representative batch (n=7 oocytes, N=1 experiment) before and after Iso. The curves were drawn using the Boltzmann equation using average $V_{1/2}$ and K_a obtained from the fits of I-V curves in individual oocytes (see Methods). Parameters used were: $V_{1/2}$=8.7 mV,

K_a=6.8 mV before Iso; $V_{1/2}$=3.8 mV, K_a=6.6 mV after Iso. **D-E**, β1-AR regulation of $Ca_V1.2$ containing the wt-α_{1C} in the presence of increasing doses of Rad. Oocytes were injected with RNAs of wt-α_{1C}, α2δ, β_{2b}, β1AR, and the indicated doses of Rad RNA. **D,** "before-after" plots of Iso-induced changes in I_{Ba} in individual cells injected with increasing doses of Rad RNA. N=1 experiment; statistics: paired t-test. **E**, Fold change increase in I_{Ba} caused by Iso as a function of Rad:β_{2b} RNA ratio. N=1 experiment. Statistics: One Way ANOVA.

Figure 6.

Figure 6. β1-AR regulation of full-length and truncated α_{1C}. A, Representative diary plots of I_{Ba} in oocytes expressing wt (full-length) α_{1C} ($Ca_V1.2$-α_{1C}) or $Ca_V1.2$-α_{1C}Δ1821 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. **B,** "Before-after" plots of Iso-induced changes in I_{Ba} in individual cells. The Rad:β2b ratio in oocytes expressing wt $Ca_V1.2$ (blue symbols) or $Ca_V1.2$Δ1821 (black symbols) was 1:3 and 1:2, respectively. Lower ratio was used in cells expressing wt-α_{1C} to allow higher basal currents. N=3; statistics: paired t-test. **C**, Rad is essential for the Iso-induced increase in I_{Ba}. N=3 experiments. Statistical analysis was performed separately for wt $Ca_V1.2$-α_{1C} and $Ca_V1.2$-α_{1C}Δ1821 with ANOVA on ranks ($p<0.001$). In addition, Mann-Whitney Rank Sum test was used to compare the last two groups, wt $Ca_V1.2$ vs. $Ca_V1.2$Δ1821 with Rad and β1-AR ($p=0.002$). For wt $Ca_V1.2$ with Rad and β1-AR, there was no significant difference from the group without β1-AR but with Rad ($p=0.076$, One-Way ANOVA on ranks, Dunnett's test).

Figure 7.

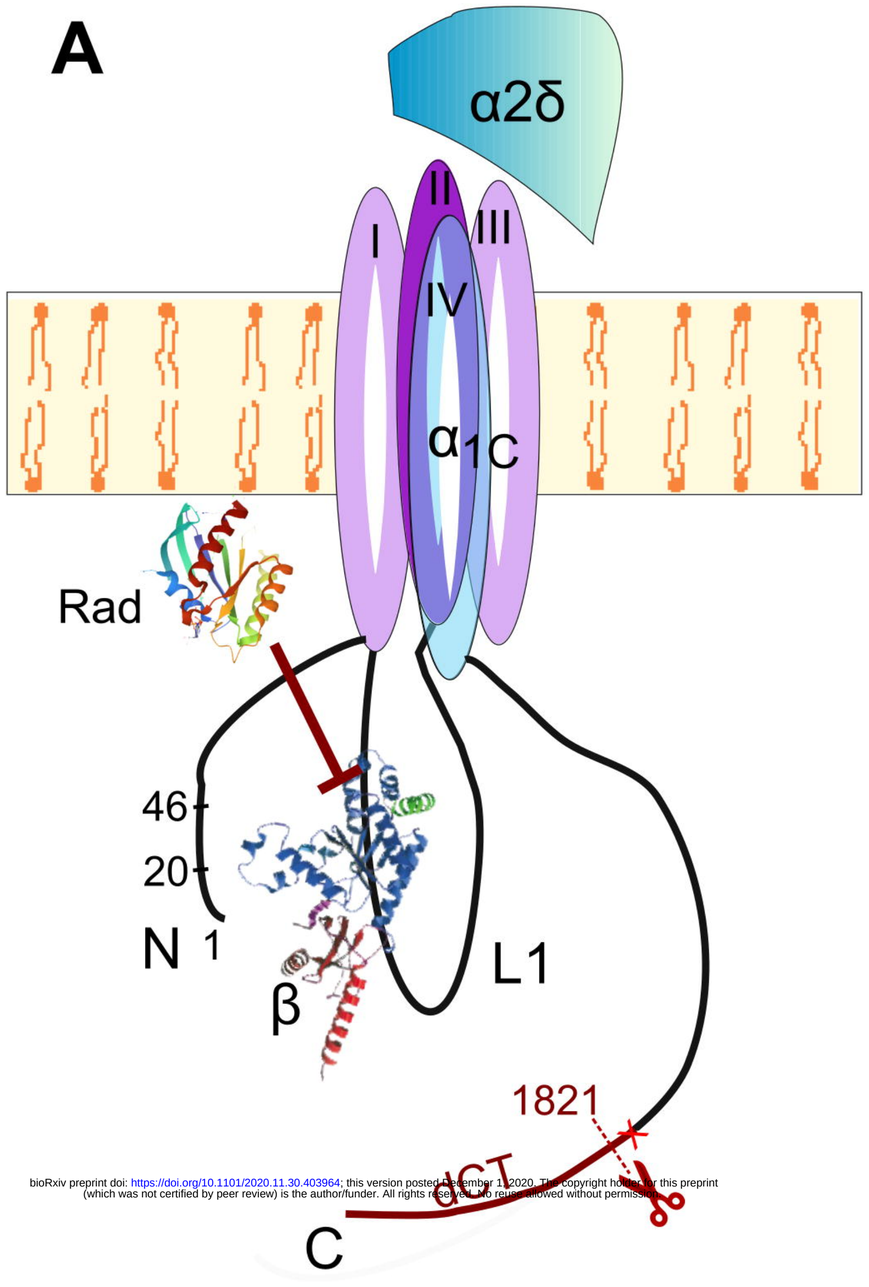
Figure 7. Comparison of β1 and β2 adrenergic regulation of $Ca_V1.2$. A, Representative diary plots of I_{Ba} showing the effect of Iso on $Ca_V1.2$-α_{1C}Δ1821 in oocytes co-expressing β1-AR (upper panel) or β2-AR (lower panel). Insets show I_{Ba} at +20 mV before and 9-10 min after the addition of Iso. RNAs of both receptors were made on the template of cDNAs inserted into the pGEM-HJ vector. **B,** "Before-after" plots of Iso-induced changes in I_{Ba} in individual cells co-expressing $Ca_V1.2$-α_{1C}Δ1821 and Rad, without any receptor or with either β1-AR or β2-AR. Data are shown

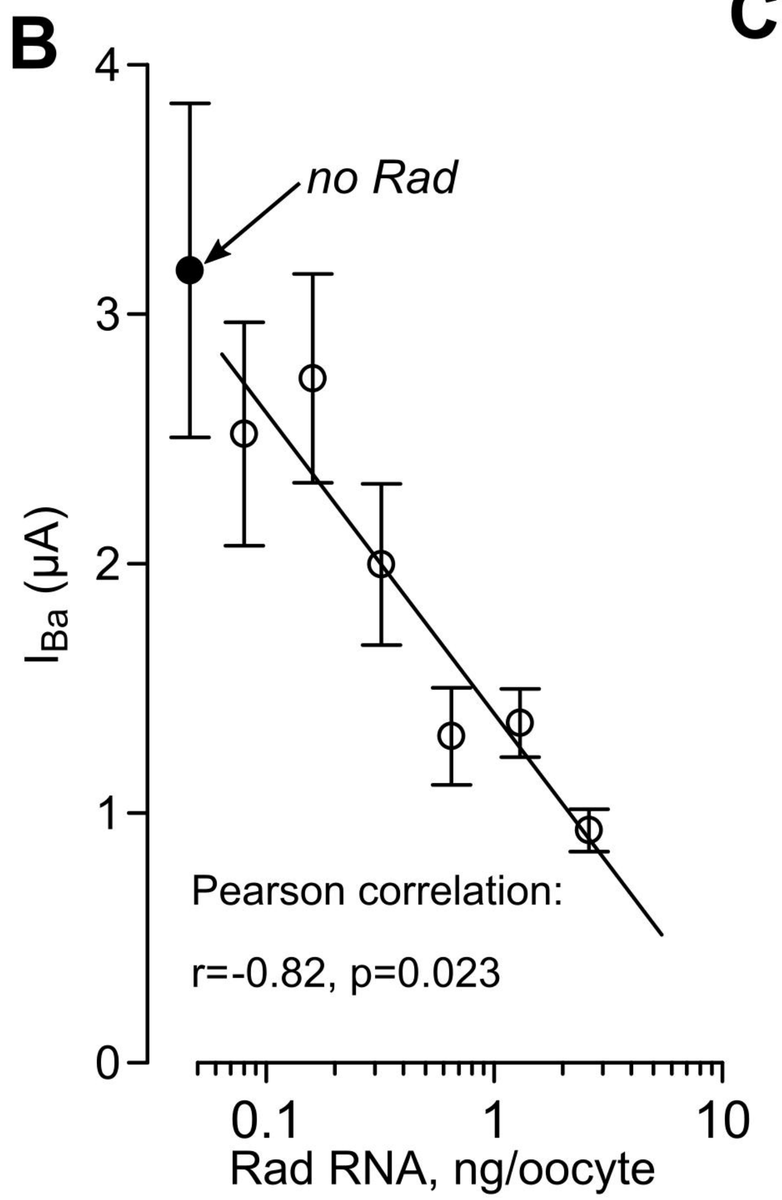
for receptors RNAs concentrations of 0, 50 and 200 pg RNA/oocyte. Rad:β_{2b} RNA ratio was 1:2. N=1 experiment; statistics: paired t-test. **C,** Fold change in I_{Ba} induced by Iso (summary of data shown in A and B). Statistics: One Way ANOVA followed by Dunnett's test.

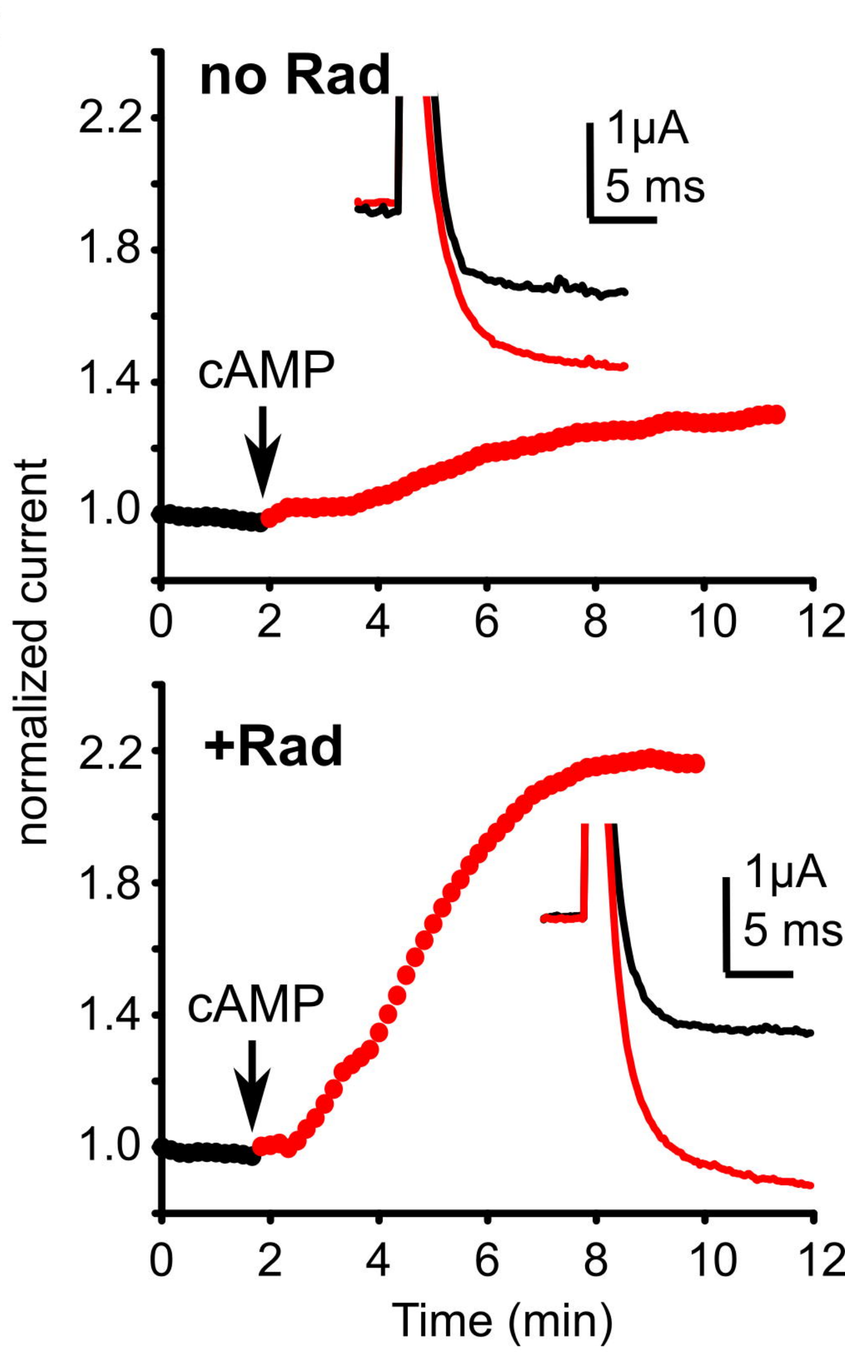
Figure 8.

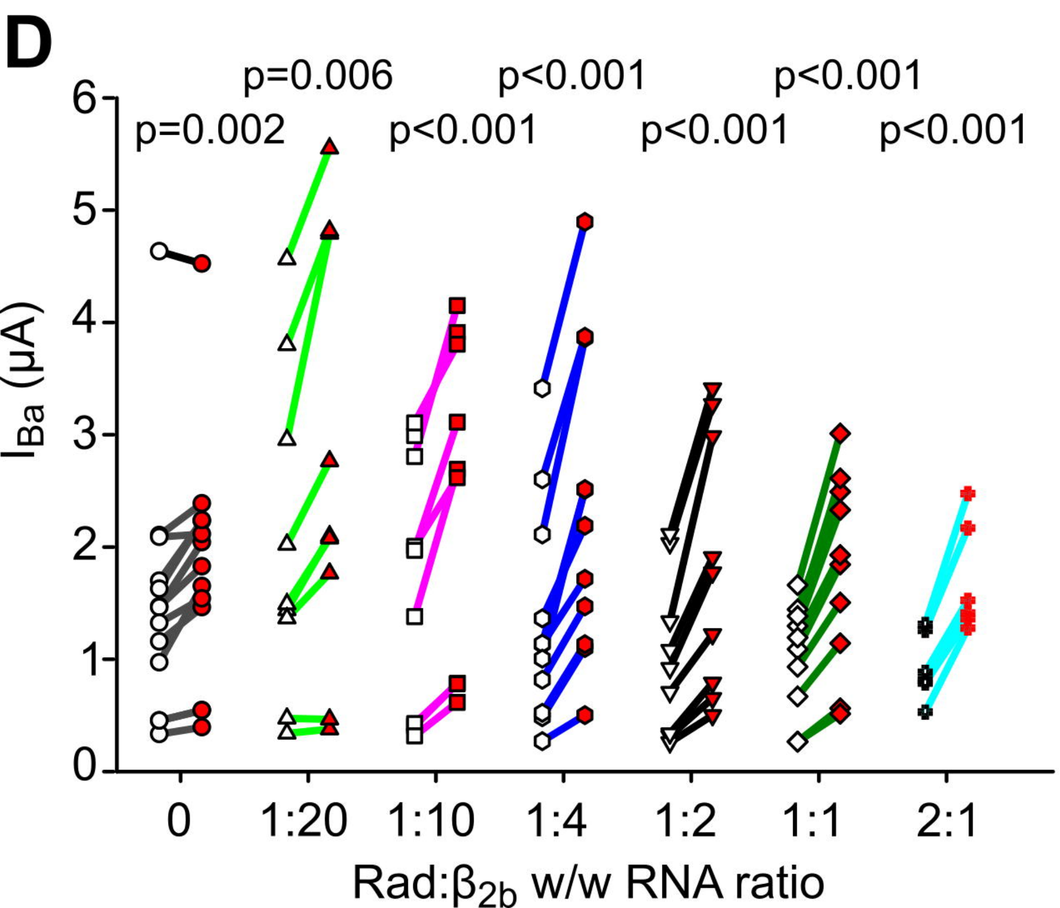
Figure 8. Reconstitution of the β2 adrenergic receptor regulation of CFTR and α_{1C}. A, Preincubation with propranolol is needed to reinstate the adrenergic regulation of CFTR by β2-AR. Oocytes expressed CFTR and β2-AR (1 and 0.005-0.05 ng/oocyte, respectively). CFTR chloride currents were recorded in gap free mode at -80 mV. 50 µm Iso was applied after stabilization of the current (about 2 min after start of record). Upper panel shows a representative record without propranolol incubation. The lower panel shows a representative oocyte preincubated in 10 µM propranolol for 60-120 min, then transferred to the experimental chamber, voltage clamped (start of record) and perfused with propranolol-free solution for about 2 min prior to the addition of Iso. **B,** Basal chloride currents in oocytes co-expressing CFTR channel (1 ng RNA/oocyte) and β2-AR (increasing doses of RNA). Oocytes expressing CFTR alone had statistically significant lower basal currents than oocytes co-expressing β2-AR (N=1 experiment; statistics: $p<0.001$, Kruskal-Wallis One Way ANOVA on Ranks) **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 Test. **D,** Comparison of basal I_{Ba} in oocytes co-expressing $Ca_V1.2$-$\alpha_{1C}\Delta1821$, Rad and β2-AR, which were incubated without propranolol (black) or with 10 µM propranolol (blue). I_{Ba} was recorded at peak current of +20 mV. Oocytes were preincubated with 10 µM propranolol for 60-120 min, then transferred to the experimental chamber, voltage clamped to +20 mV and perfused with propranolol-free 40 mM Ba^{2+} solution until stabilization of the current for 2 min. Propranolol washout lasted approximately 1-2 minutes. Oocytes co-expressed $\alpha_{1C}\Delta1821$, β_{2b}, α2δ, Rad (Rad:$\beta2_b$ RNA ratio was 0.5). N=1; statistics: t-test. **E-F**, Representative diary plots of oocytes co-expressing $\alpha_{1C}\Delta1821$, Rad and β2-AR without (left) and with (right) preincubation with propranolol. **G,** "Before-after" plots of Iso-induced changes in I_{Ba} in individual cells. Blue

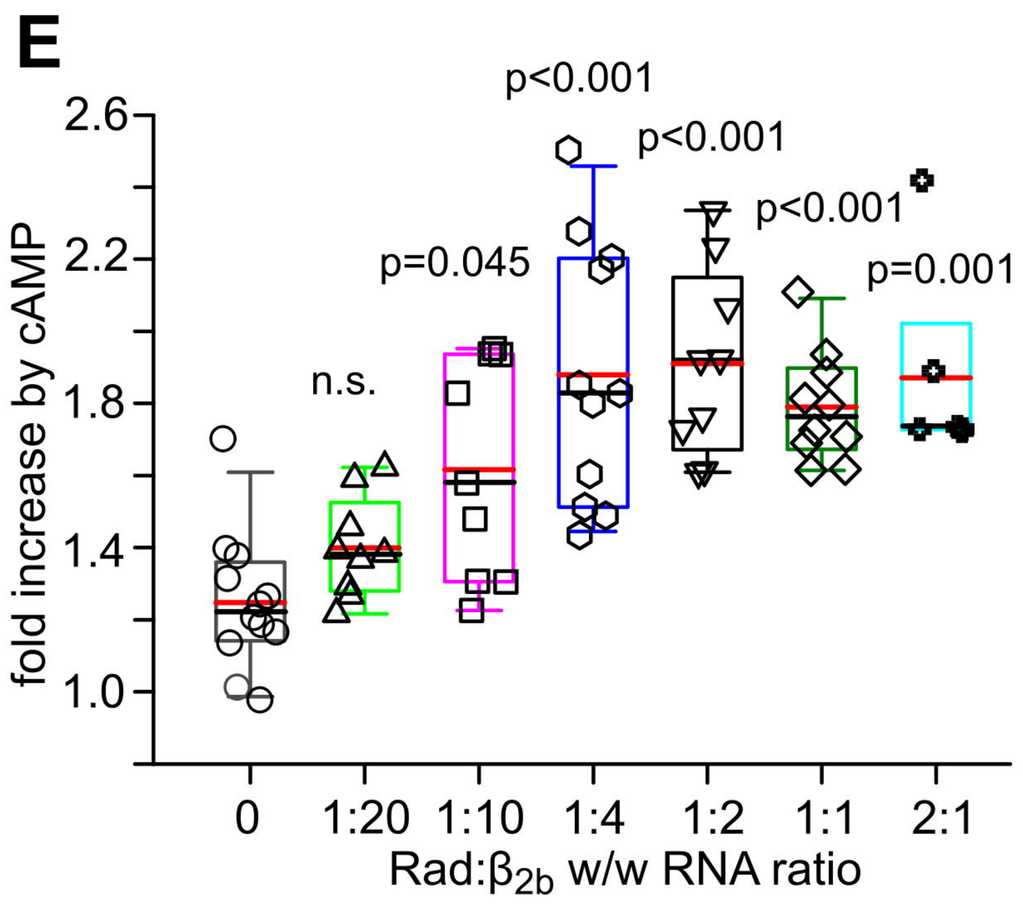
symbols represent data form oocytes preincubated with propranolol, black – without preincubation. N=1; statistics: paired t-test. **H,** Fold increase in I_{Ba} induced by Iso (summary of data shown in G). Oocytes preincubated with propranolol (blue) had statistically significant higher fold increase. N=1; statistics: Kruskal-Wallis One Way ANOVA on Ranks followed by Dunnett's test.

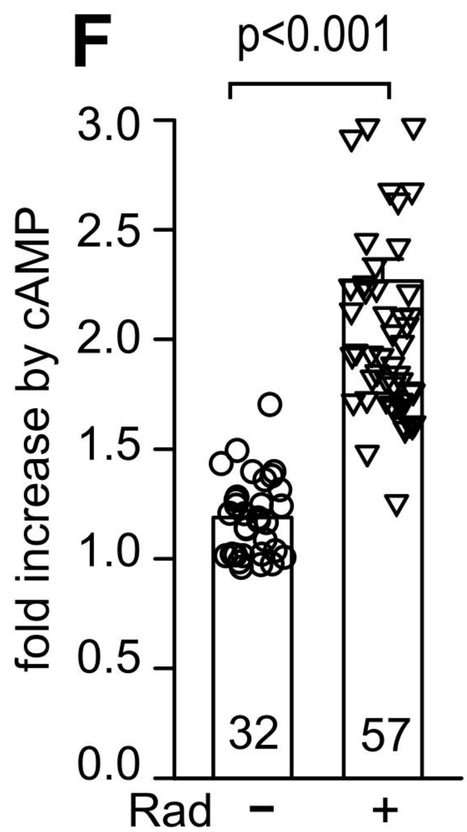


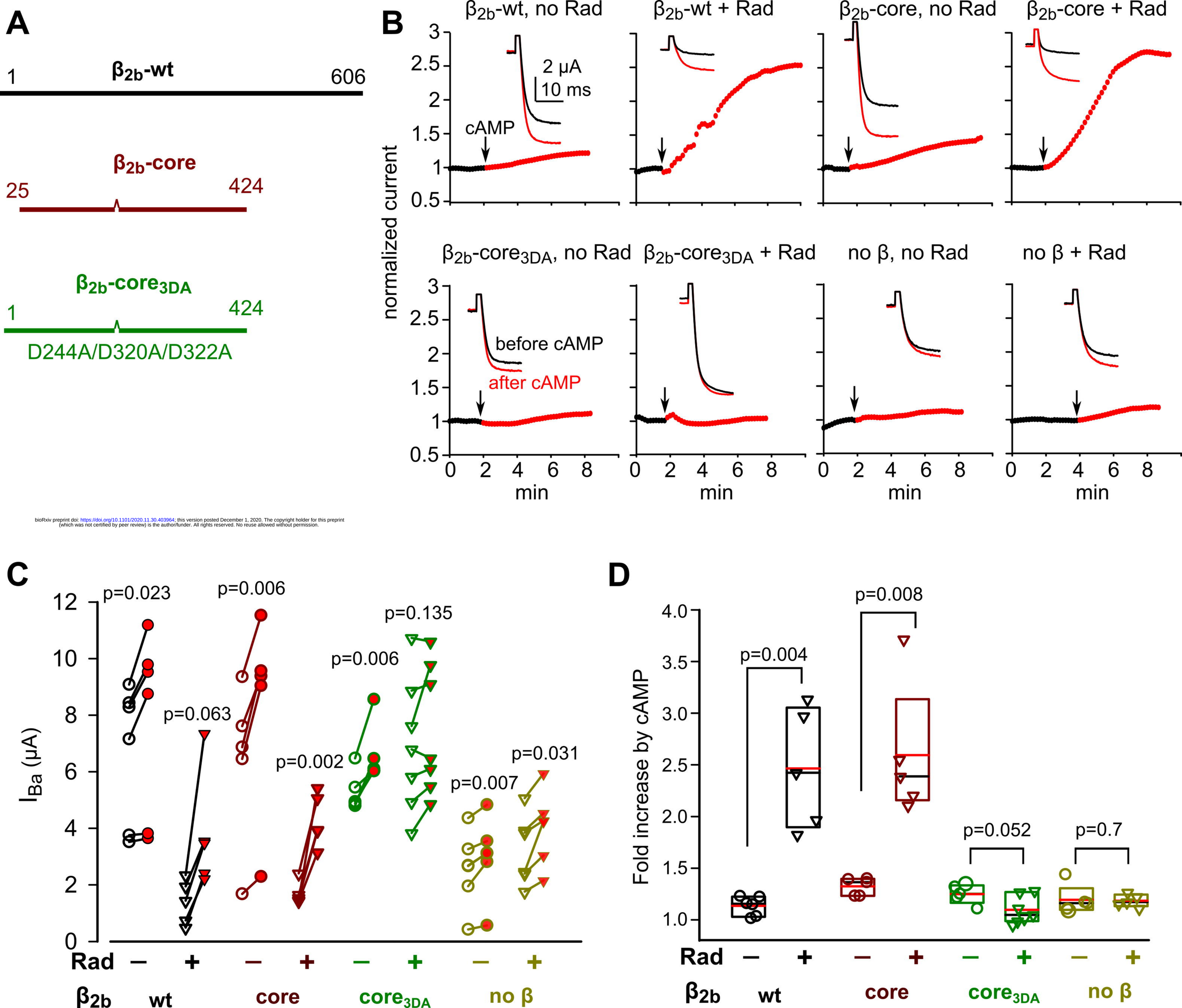


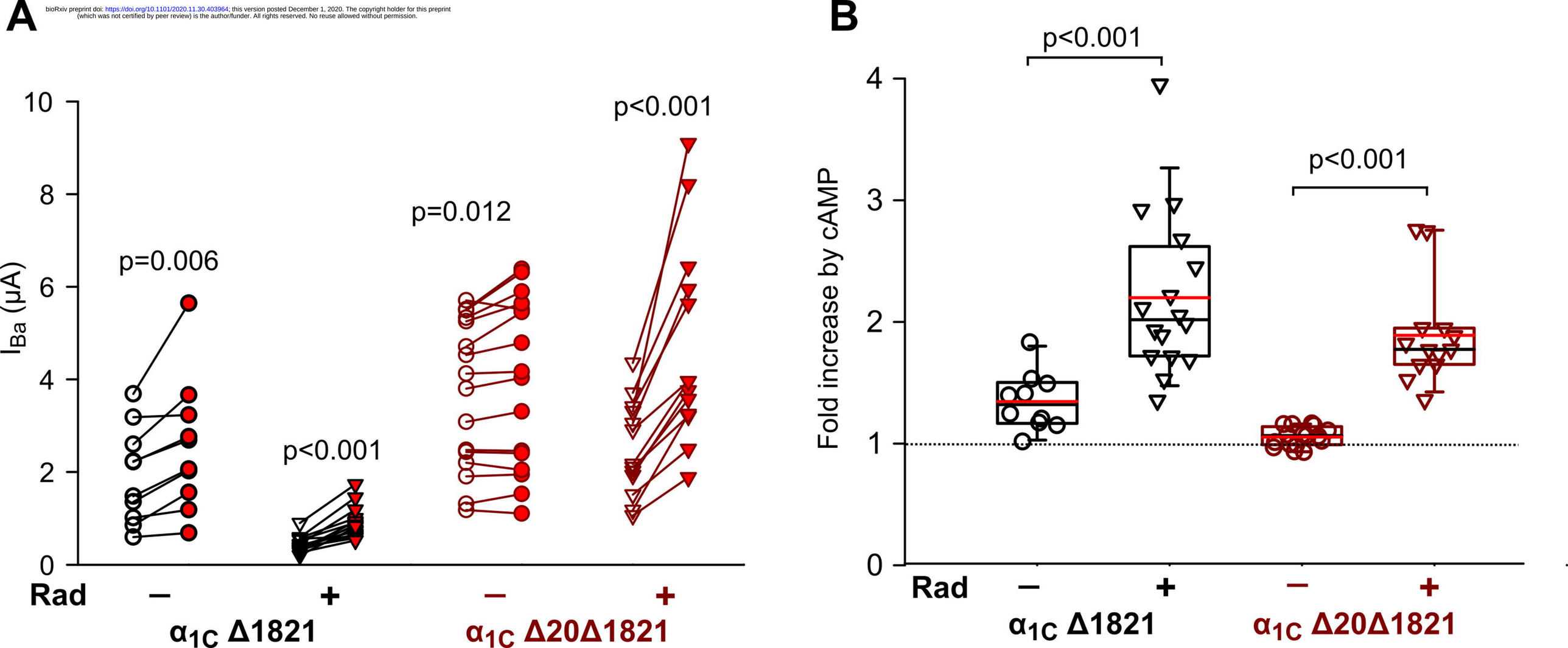


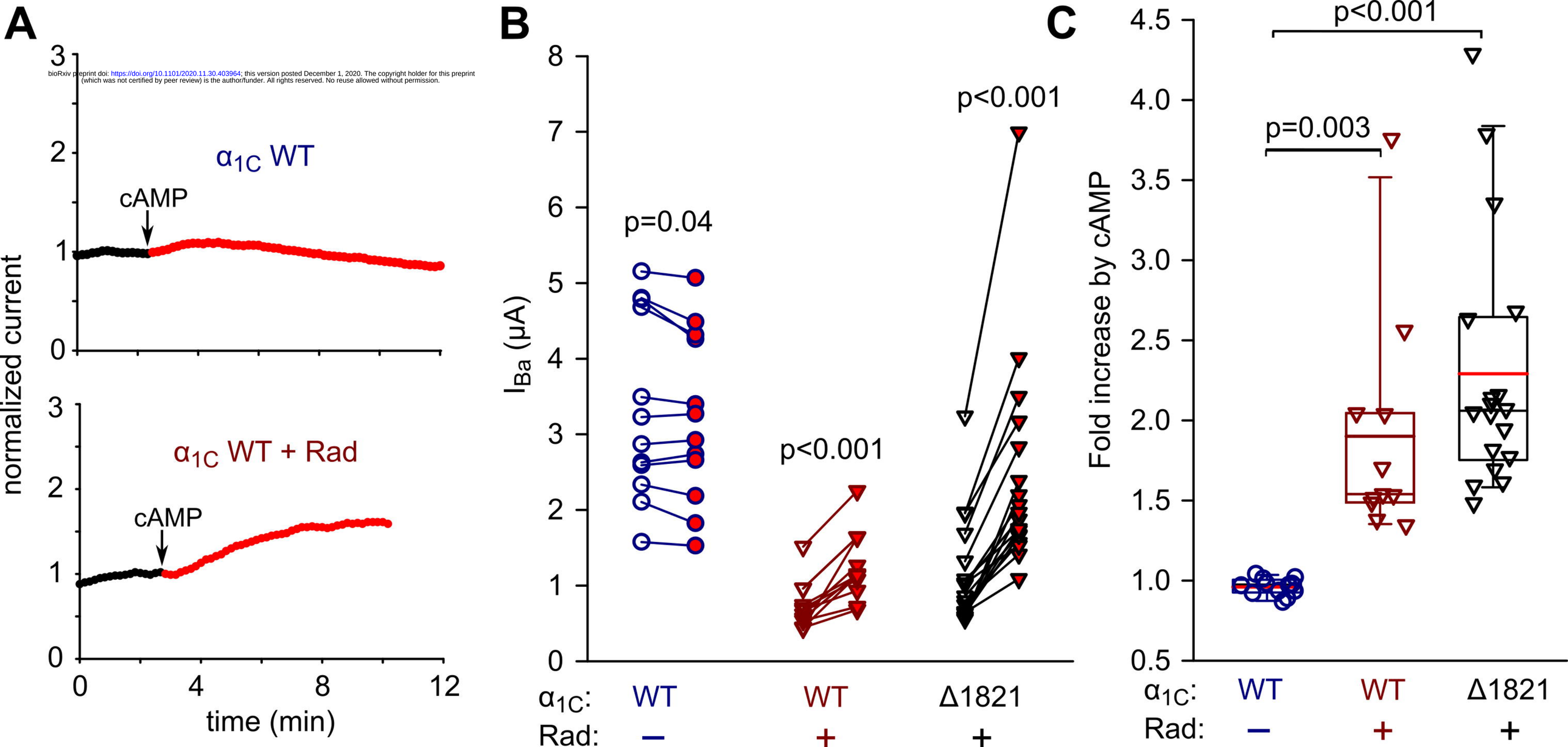


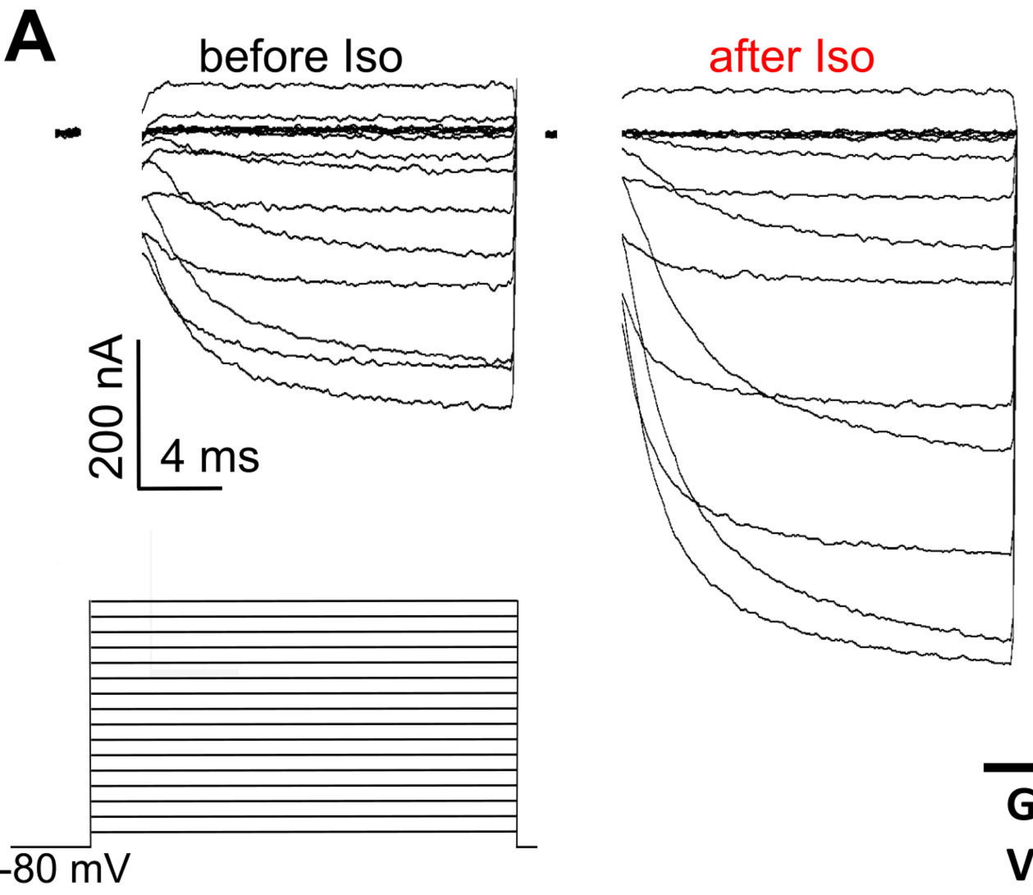


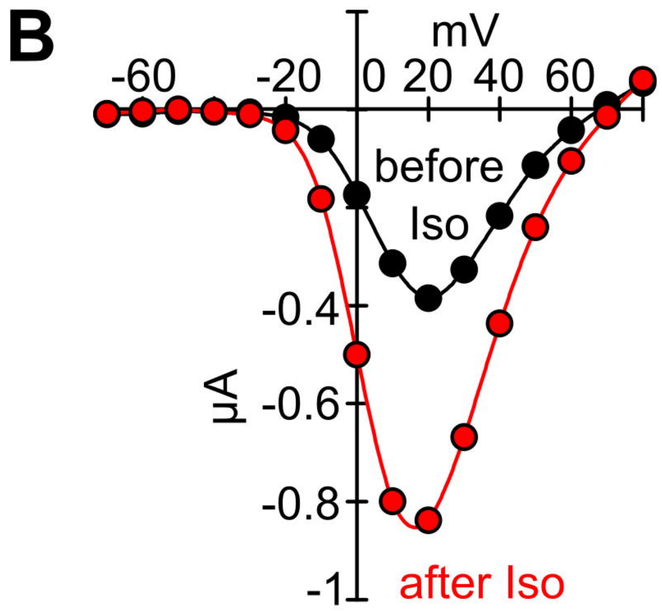


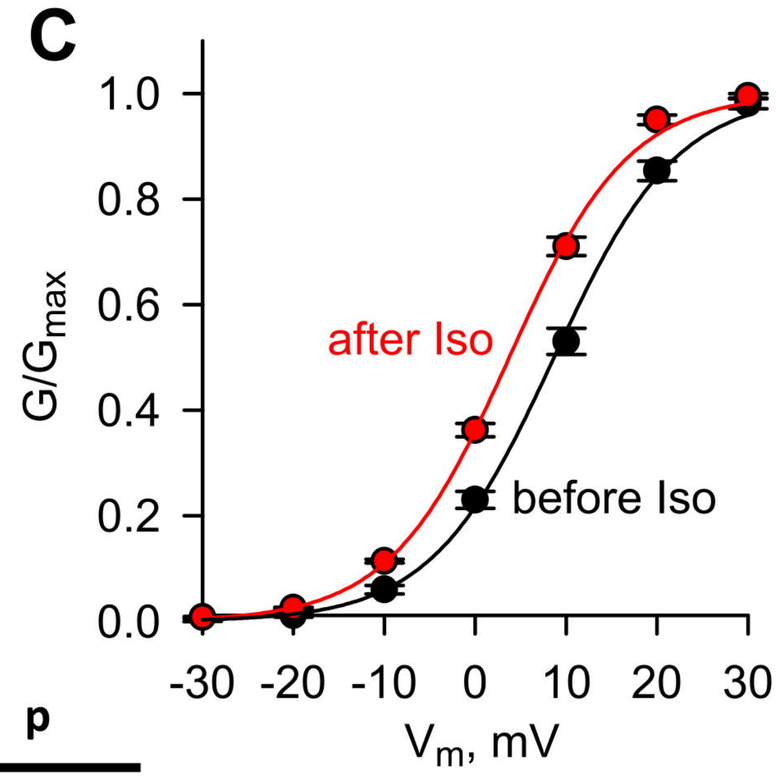




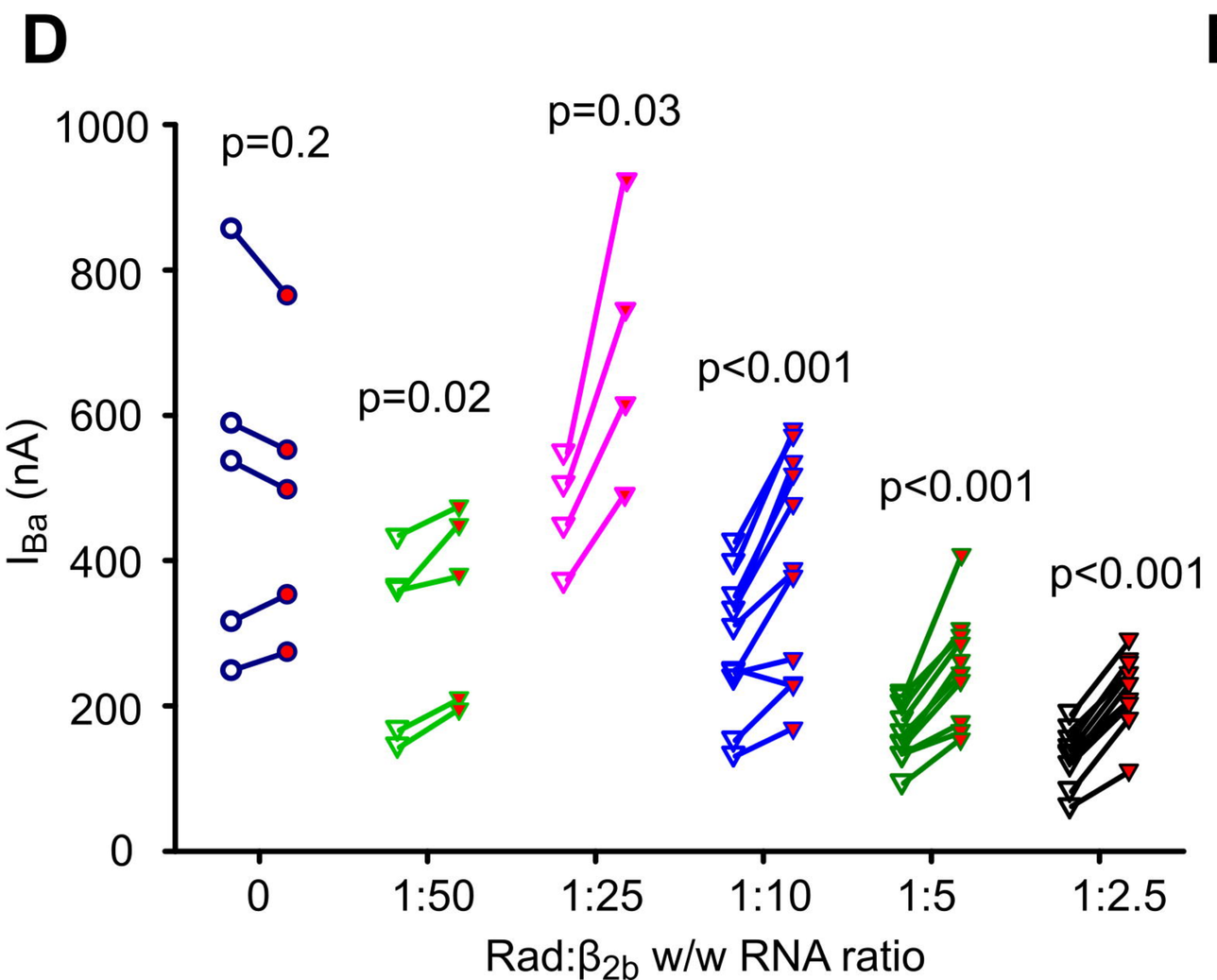


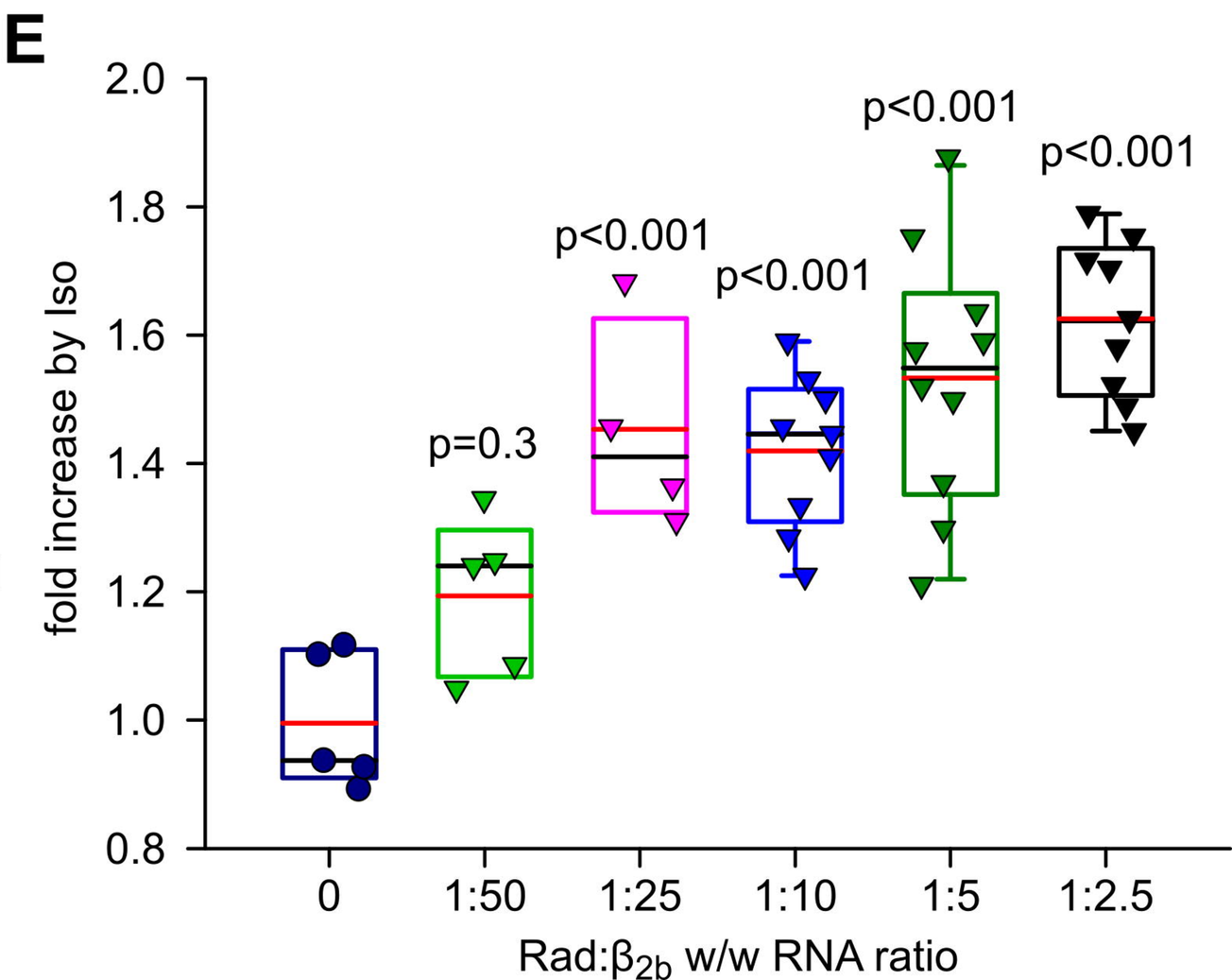






	Before Iso	After Iso	p
Gmax	6.9±0.9	17.8±1.5	<0.001
V_{rev}	61±1.4	64±0.3	0.075
$V_{1/2}$	8.7±0.7	3.8±0.4	0.016
K_a	6.8±0.13	6.6±0.14	0.3





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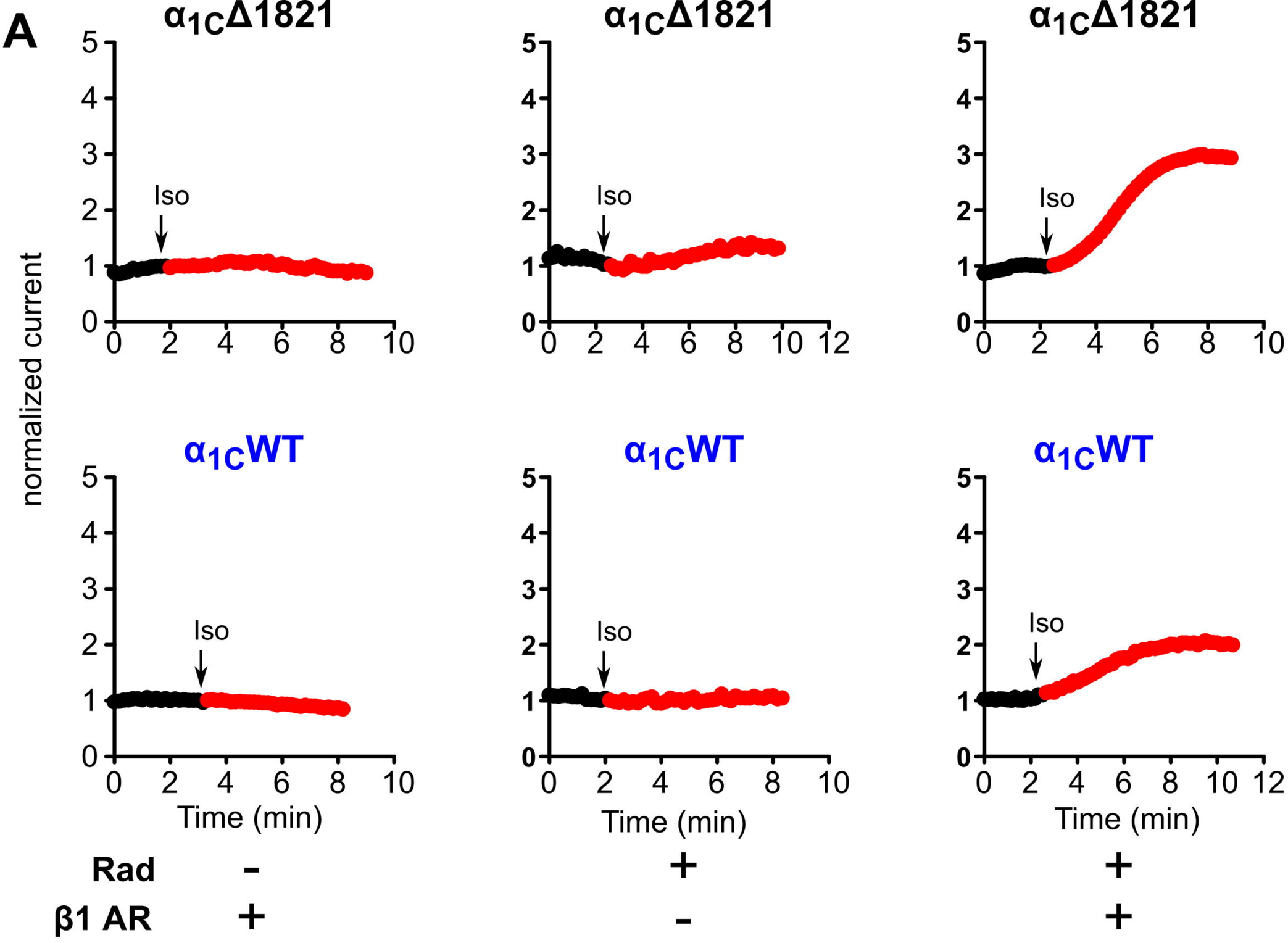

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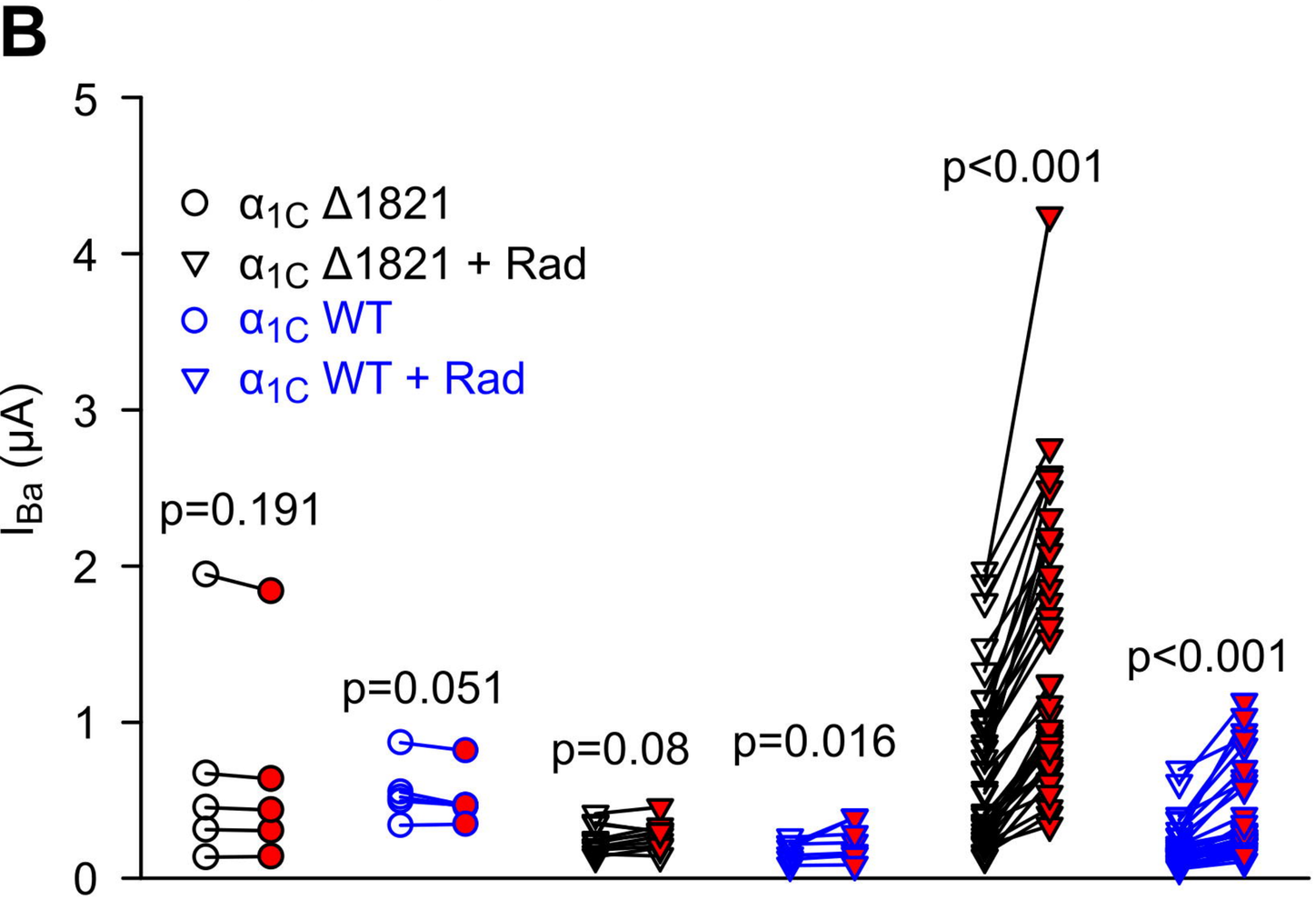

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