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Carvedilol inhibits cADPR- and IP_3-induced Ca^{2+} release

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Abstract

Spontaneous Ca^{2+} waves, also termed store-overload-induced Ca^{2+} release (SOICR), in cardiac cells can trigger ventricular arrhythmias especially in failing hearts. SOICR occurs when RyRs are activated by an increase in sarcoplasmic reticulum (SR) luminal Ca^{2+}. Carvedilol is one of the most effective drugs for preventing arrhythmias in patients with heart failure. Furthermore, carvedilol analogues with minimal β-blocking activity also block SOICR showing that SOICR-inhibiting activity is distinct from that for β-block. We show here that carvedilol is a potent inhibitor of cADPR-induced Ca^{2+} release in sea urchin egg homogenate. In addition, the carvedilol analog VK-II-86 with minimal β-blocking activity also suppresses cADPR-induced Ca^{2+} release. Carvedilol appeared to be a non-competitive antagonist of cADPR and could also suppress Ca^{2+} release by caffeine. These results are consistent with cADPR releasing Ca^{2+} in sea urchin eggs by sensitizing RyRs to Ca^{2+} involving a luminal Ca^{2+} activation mechanism. In addition to action on the RyR, we also observed inhibition of inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release by carvedilol suggesting a common mechanism between these evolutionarily related and conserved Ca^{2+} release channels.

Introduction

Calcium (Ca^{2+}) is a highly versatile intracellular signal that regulates a wide range of cellular functions, including exocytosis, contraction, metabolism, transcription, fertilization and cell proliferation1. Sources for Ca^{2+} signaling include both influx through channels at the plasma membrane and Ca^{2+} mobilization from Ca^{2+} storage organelles. A key question is how the ubiquitous Ca^{2+} signaling ion can control cellular processes with a high degree of specificity. One potential solution is the realization that there are multiple Ca^{2+} mobilizing messengers targeting specific Ca^{2+} release channels in distinct organelles. Three molecules have satisfied all the criteria for assignment as Ca^{2+} mobilizing second messengers. These are IP_3, and the pyridine nucleotide metabolites, cADPR and NAADP 2. The first two molecules target Ca^{2+} release channels in the cell's largest Ca^{2+} storage organelle, the

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endoplasmic reticulum (ER), whilst NAADP releases Ca^{2+} from organelles of the endolysosomal system likely through two-pore channels (TPCs) 3, 4.

IP_3R and RyR channels are the evolutionarily related principal sarco-endoplasmic Ca^{2+} release channels mediating Ca^{2+} mobilization from this organelle in response to various stimuli 5. These channels are the main mediators of *Ca^{2+}-induced Ca^{2+}release* (CICR) 6, an autocatalytic mechanism by which cytoplasmic Ca^{2+} activates the release of Ca^{2+} from internal stores. This mechanism contributes to the globalization of intracellular Ca^{2+} signals in cells including propagating Ca^{2+} waves, since in the absence of such mechanisms buffering mechanisms greatly restrict the spatial diffusion of Ca^{2+} signals making them inherently local. IP_3 and cADPR are thought to evoke openings of channels by sensitizing them to Ca^{2+} as a co-agonist 6. Two principal modes for triggering CICR have been proposed. The first is a cytosolic mode whereby an increase in cytosolic Ca^{2+} may activate Ca^{2+} release channels. The second is a luminal mode whereby an increase in intraluminal Ca^{2+} concentrations trigger the opening of Ca^{2+} release channels. The latter is associated with the phenomenon of spontaneous Ca^{2+} release from ER/SR7–9, which has been proposed as an important mechanism underlying various cardiac arrhythmias 10.

cADPR was first identified as a Ca^{2+} mobilizing molecule in sea urchin eggs and homogenates 11. Previous work had indicated that NAD was enzymatically converted to a Ca^{2+} mobilizing agent in sea urchin eggs and homogenates 12. Pharmacological studies showed that cADPR targeted RyRs but not IP_3Rs based on cross-desensitization with RyR activators and inhibition by RyR blockers 13. Furthermore, both divalent cations and the RyR pharmacological activator caffeine, potentiate Ca^{2+} release by cADPR 14. Subsequent studies in many mammalian cell types support the link between cADPR and RyRs and, in cardiac cells, cADPR promotes the production of Ca^{2+} sparks and regulates contractility 15 16. An excess of cADPR may be pro-arrhythmic 16, and an inhibitor of the enzyme that synthesizes cADPR is proposed as a novel anti-arrhythmic drug 17. In summary, cADPR is considered a second messenger that acts by sensitizing RyRs to CICR 18.

It has long been recognized that spontaneous Ca^{2+} release occurs during SR Ca^{2+} overload, a process also known as store-overload induced Ca^{2+} release (SOICR). This is a luminal mechanism whereby as the ER (or SR) fills with Ca^{2+} when the intra-luminal Ca^{2+} concentration or amount reaches a certain level or threshold, the opening of RyRs is triggered 10. Such mechanisms have been suspected from studies on spontaneous Ca^{2+} release from the SR 7–9, but Chen and his colleagues demonstrated a drug that could block the process 19 and also pinpointed the molecular mechanism by which luminal Ca^{2+} triggers SOICR 10. Such spontaneous Ca^{2+} release may lead to propagating Ca^{2+} waves, essential for activation of eggs at fertilization, but potentially fatal in cardiac myocytes since they underlie arrhythmias 20. Chen and his colleagues proposed that, uniquely amongst β-blockers, carvedilol suppresses SOICR itself, in addition to its β-blocking action 19. Luminal amino-acid residues of the RyR have been identified as critical for SOICR, and these include the E4872 residue, which is highly conserved not only in RyR between species but also in IP_3Rs 10. The proposal is that the RyR itself acts as a luminal Ca^{2+} sensor, rather than an accessory or interacting protein such as calsequestrin, as SOICR can persist in

calsequestrin-null mice 21. The E4872Q mutation in a mouse model suppressed Ca^{2+} waves and ventricular tachyarrhythmias (VTs) 10.

It has been shown that, compared to 14 other known β-blockers, carvedilol was the only one effective in inhibiting SOICR 19. This unique inhibitory action of carvedilol on SOICR needs further investigation, as this activity would be contributing to its β-blocking action. Single-channel recordings in lipid bilayers revealed that carvedilol has an impact on the gating properties of RyR2, reducing the duration and increased the frequency of channel openings. In this study, we have utilized the sea urchin egg homogenate preparation 12 which robustly express both IP_3R and RyR Ca^{2+} release mechanisms on microsomal vesicles 22. Furthermore, ER-derived microsomes in sea urchin egg homogenates display spontaneous Ca^{2+} release due to Ca^{2+} overload, which can be effected by both IP_3Rs and RyRs 9. This allowed us to examine the effect of carvedilol and analogs on SOICR mediated by the two principal ER-based Ca^{2+} release mechanisms. Although the interaction of IP_3 with IP_3Rs has now been elucidated at the molecular level 23, the mechanism by which cADPR activates RyRs is not well understood, with accessory cADPR binding proteins proposed 24–26. However, since cADPR is known to cause Ca^{2+} release by modulating RyRs 13, 14, it allows us to probe the role of SOICR in the mechanism by which cADPR activates RyRs. We find that carvedilol suppresses cADPR-evoked Ca^{2+} release, and also attenuates IP_3-mediated Ca^{2+} release. A common mechanism by which carvedilol inhibits a conserved luminal Ca^{2+} gate for both Ca^{2+} release mechanisms is suggested.

Materials and Methods

Sea urchin egg homogenate preparation

Gamete shedding was stimulated by intracoelomic injections of 0.5 M KCl solution and sea urchin eggs from *Lytechinus pictus* were collected. Collection was carried out in artificial sea water (ASW). ASW: 435 mM NaCl, 10 mM KCl, 40 mM $MgCl_2$, 15mM $MgSO_4$, 11 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 7 mM Tris base, 13 mM Tris-HCl, pH 8.0. Eggs were de-jellied by passage through 100 μM nylon mesh (Millipore), and then washed 4 times in Ca^{2+}-free ASW, with the first two washes containing 1 mM EGTA. Eggs were subsequently washed in intracellular-like medium, glucamine intracellular medium (GluIM). GluIM: 250 mM potassium gluconate, 250 mM N-methyl-D-glucamine, 20 mM Hepes (acid), and 1 mM $MgCl_2$, pH 7.2 (pH adjusted with glacial acetic acid). Eggs were homogenized with a glass Dounce tissue homogenizer in ice-cold GluIM supplemented with 2 mM MgATP, 20 U/mL creatine phosphokinase (CPK), 20 mM phosphocreatine (PCr), Complete ™EDTA-free Protease Inhibitor tablets were from Roche. Homogenate (50%, v/v) was then centrifuged at 13,000 x g at 4 °C for 10 s. The supernatant were aliquoted into 0.5 ml portions and stored at -80 °C.

Fluorimetry to measure Ca^{2+} release

To measure Ca^{2+} changes, egg homogenate was diluted gradually 20-fold over 3 h in GluIM at 17°C supplemented with the ATP regenerating system (to facilitate optimal Ca^{2+} loading of the stores), fluorescent dyes were added (Fluo-3, 3 μM final) and fluorescence changes

monitored in a 600 μl aliquot (2.5% v/v) in a stirred micro-cuvette in a Perkin Elmer LS-50B fluorimeter.

Fluo-3 was excited at 506 ± 3 nm and 526 ± 2 nm emission recorded.

The Fluo-3 fluorescence signal was converted to absolute Ca^{2+} concentrations by sequential addition of 0.5 mM EGTA and 10 mM $CaCl_2$ to determine F_{min} and F_{max}, using the following equation:

$$\left[Ca^{2+}\right] = \left[K_d \times (F - F_{min})\right] / (F_{max} - F_{min})$$

where $[Ca^{2+}]$ is the extravesicular Ca^{2+} concentration, F is the fluorescence at any time, F_{min} and F_{max} are the fluorescence in the "absence" of Ca^{2+} and presence of saturating Ca^{2+}, respectively. At the end of each experiment, F_{min} and F_{max} were empirically determined by the sequential addition of 500 μM EGTA and 10 mM Ca^{2+}, respectively (6 μL of 50 mM EGTA; 6 μL of 1 M $CaCl_2$).

For TPEN studies of effects on the kinetics of Ca^{2+} release, fluorescence (in units (U)/s) was normalized to the resting fluorescence (F_0) to account for variability and therefore expressed as $U.F_0/s$.

Source of reagents

cADPR and IP_3 were purchased from Sigma-Aldrich and LC Laboratories, respectively. Fluo-3 (K^+ salt) was purchased from Invitrogen and TPEN from Sigma-Aldrich. Carvedilol was purchased from Abcam Biochemicals. VK-II-86, the carvedilol analog, was synthesized as described previously19.

Statistical analysis

Two data sets were compared using Student's t test and multiple groups using ANOVA and Dunnett's multiple comparisons post test. Data were paired when needed and statistical significance assumed at $P<0.05$. Graphs were constructed and the following statistical conventions observed: $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***), $P<0.0001$ (****) using Graphpad Prism. Representative fluorimetric traces were plotted as raw fluorescence (RFU) against time.

Results

To gain further insight into the mechanism of cADPR-induced Ca^{2+} release, we examined the effect of carvedilol on Ca^{2+} release mechanisms in in sea urchin egg homogenates. cADPR-induced Ca^{2+} release was monitored in the presence of vehicle DMSO or carvedilol. A sub-maximal concentration of cADPR (0.3 μM) for Ca^{2+} release was determined by establishing a concentration-response curve of cADPR (EC_{50} ~ 0.25 μM; Fig. 2b). Preincubation with carvedilol for 2 minutes resulted in a concentration-dependent inhibition of cADPR-induced Ca^{2+} release with an approximate IC_{50} of 30 μM (Fig. 1b).

Generating a cADPR concentration-response curve in the presence or absence of a sub-maximal concentration of carvedilol revealed that carvedilol reduced the maximal response to cADPR without substantially changing the EC_{50} for cADPR (Fig. 2b), i.e. was likely a non-competitive blocker. The EC_{50} of cADPR with DMSO (and 95% confidence interval) was 250 nM (153–391 nM) and in the presence of carvedilol it was 410 nM (297–564 nM) ($P>0.05$).

Next we examined the time course of inhibition by carvedilol. Carvedilol (30 μM) was preincubated with homogenate for 10, 30, 60 and 120 seconds. Our results show that carvedilol suppressed cADPR-induced Ca^{2+} release maximally within 10 seconds, and there was no further inhibition at longer incubations (Fig. 3).

In order to investigate whether the effect of carvedilol was unique to cADPR- induced Ca^{2+} release and the RyR, we examined the effects of carvedilol on IP_3-induced Ca^{2+} release. The proposed SOICR trigger of RyRs has been proposed to be conserved in IP_3Rs 10. Carvedilol also suppressed IP_3-induced Ca^{2+} release but the extent of inhibition of IP_3 was less than that for cADPR (Fig. 4).

Caffeine is also a commonly used pharmacological RyR agonist 27. Caffeine has also shown to release Ca^{2+} in sea urchin egg homogenates and eggs by activating RyRs 13, 28. We compared the effects of carvedilol on cADPR- and caffeine-induced Ca^{2+} release. Different concentrations of carvedilol were preincubated for 2 minutes prior to the addition of 10 mM caffeine13 or 0.3 μM cADPR (Fig. 5a). Carvedilol suppressed caffeine-induced Ca^{2+} release with an IC_{50} indistinguishable from to cADPR with IC_{50}s of 45 μM (for cADPR) and 51 μM for caffeine. The differences were not statistically significant.

VK-II-86 is a carvedilol analog but with minimal β-blocking activity, demonstrating that the pharmacophore for SOICR inhibition can be separated from that for β-blockade 19, 29. Like Carvedilol, VK-II-86 inhibited cADPR-induced Ca^{2+} release in a concentration-dependent manner (IC_{50} of 52 μM; Fig. 6b), demonstrating that the β-blocking activity of carvedilol was dispensable for blocking Ca^{2+} release in homogenates.

To investigate the proposed luminal mechanism of action of carvedilol, the membrane-permeant low affinity Ca^{2+} chelator TPEN (N,N,N',N'- tetrakis(2-pyridylmethyl)ethylenediamine) was employed to allow the manipulation of luminal Ca^{2+} as previously shown 30. Different concentrations of TPEN (or ethanol vehicle) were preincubated with homogenates for 2 minutes prior to the addition of 0.3 μM cADPR (Fig. 7a). We found that TPEN inhibited cADPR-induced Ca^{2+} release, both in terms of the magnitude and kinetics of response but have a greater impact on kinetics (estimated carvedilol IC_{50} of 9 μM and 663 μM for kinetics (Fig. 7c) and magnitude respectively (Fig. 7b). The inhibition by TPEN is consistent with a perturbation of luminal Ca^{2+} as previously observed for IP_3 30. However, the effect of TPEN and carvedilol are dissimilar, as the kinetic data may indicate an additional site of action for carvedilol, and which at high concentrations almost completely inhibits Ca^{2+} release in response to cADPR.

Discussion

In the present study we have demonstrated that carvedilol inhibits cADPR-induced Ca^{2+} release in sea urchin egg homogenates. cADPR has been characterized as a second messenger regulating the sensitivity of RyRs to activation by Ca^{2+} in sea urchin eggs 13, 14, and widely in plant and animal cells 2. However, the mechanism of action of cADPR, from sea urchin egg to cardiac cells, remains undefined 31. A possible mechanism is for cADPR to activate the RyR indirectly by binding to a protein that is part of the RyR macromolecular complex or to a protein that either translocates itself to the RyR or influences a signalling pathway that regulates RyR activity 26. Another possible mechanism is that cADPR stimulates an increase in the level of SR Ca^{2+}, perhaps with the involvement of SERCA 31, resulting in a Ca^{2+} overload response. Due to the recent elucidation of the action of carvedilol actions on RyRs, we tested the effect of this drug on cADPR-induced Ca^{2+} release in sea urchin egg homogenates. We found that carvedilol was an effective inhibitor of cADPR-induced Ca^{2+} release. Carvedilol caused a substantial inhibition (82%) of cADPR-induced Ca^{2+} release (Fig. 1). This is unlikely to be due to blocking the cADPR binding site since carvedilol inhibition is likely non-competitive, and carvedilol also inhibits caffeine and also IP_3-induced Ca^{2+} release. It is known that although cADPR, caffeine and ryanodine can release Ca^{2+} by activating RyRs, a selective inhibitor of cADPR, 8-NH_2-cADPR does not block Ca^{2+} release by caffeine or ryanodine implying action at different but interacting sites 32. Therefore we propose also that carvedilol and cADPR act at distinct sites.

VK-II-86 also was found to suppress cADPR-induced Ca^{2+} release and this is consistent with the notion that the effect of carvedilol on cADPR-evoked Ca^{2+} release is independent of its β-blocking activity.

It has been proposed that carvedilol inhibits spontaneous Ca^{2+} release from the SR by a mechanism dependent on a luminal trigger site on RyRs. Amino acid residues important for such triggering have been identified and include a cluster of negatively charged residues at the C-terminal part of the predicted inner helix of the RyR 10. These include the residue E4872 conserved between RyRs, including that for sea urchins (Fig. 8). Importantly, acidic residues at the putative luminal sensor are also conserved in sea urchin IP_3Rs. We have shown for the first time that carvedilol also inhibits IP_3-induced Ca^{2+} release in urchin homogenates (Fig. 4), although to a lesser degree (47% versus >80%) than that for either cADPR or caffeine-induced Ca^{2+} release. Since IP_3-mediated Ca^{2+} signaling has also been implicated in arrhthymogenesis 33, this may also be pertinent to the clinical effects of carvedilol. Our novel finding that carvedilol can suppress IP_3-induced Ca^{2+} release also suggests that carvedilol may be a potential therapeutic agent for treating other IP_3R-associated disorders in addition 34.

A luminal Ca^{2+} regulation site is consistent with the inhibition of a low affinity membrane-permeant Ca^{2+} chelator, TPEN. Not only do IP_3Rs and RyRs share the conserved acidic residues, but they also share sensitivity to TPEN 30 (and this study). Moreover, since TPEN by modulating luminal Ca^{2+} similarly inhibits Ca^{2+} release by both IP_3 and cADPR in a similar way to carvedilol, a common mechanism based on a luminal mechanism is suggested. We previously have proposed that TPEN does not simply reduce the

electrochemical gradient for Ca^{2+} because ionomycin-evoked Ca^{2+} release is relatively insensitive to TPEN 30. Again, this implies a modulatory role of TPEN on Ca^{2+} release channels by lowering luminal Ca^{2+}, whereas carvedilol has a similar effect by blocking the luminal Ca^{2+} sensor on Ca^{2+} release channels.

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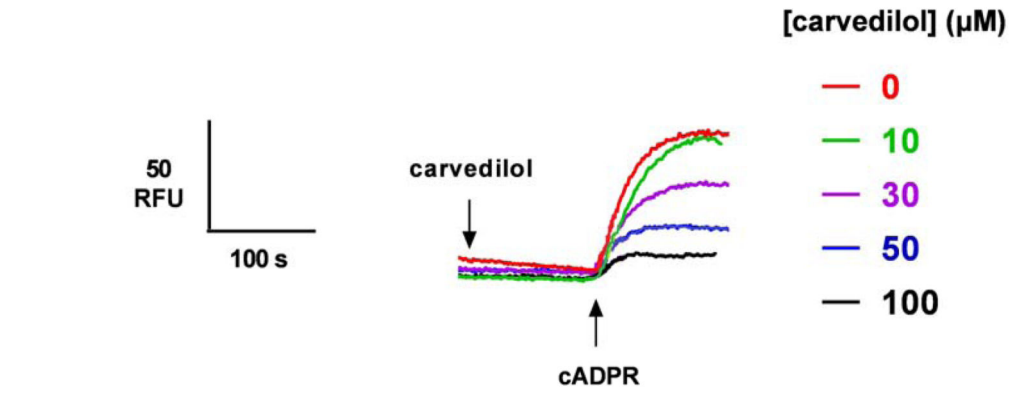

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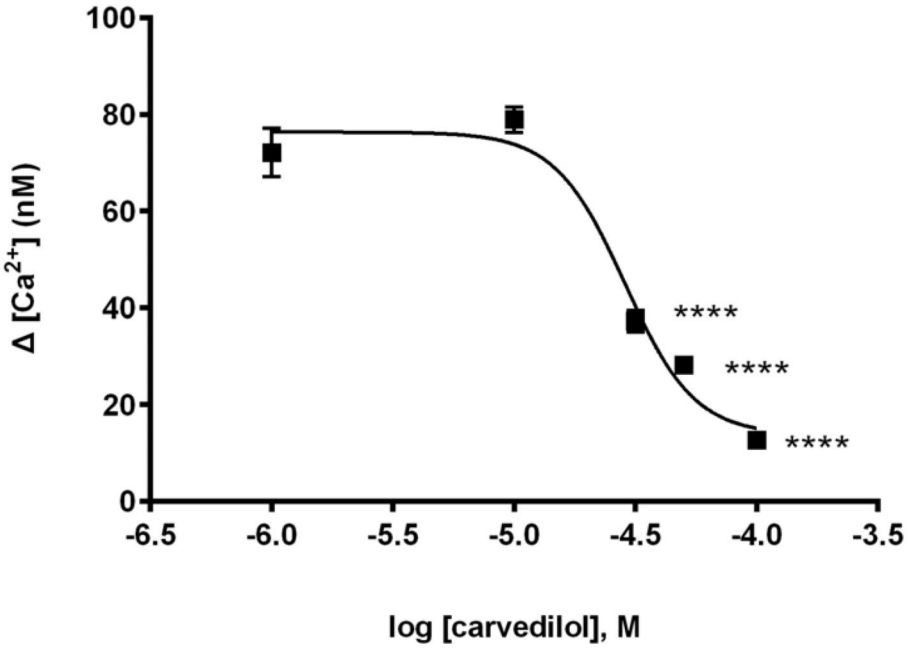

(b)

Fig. 1. Carvedilol suppresses cADPR-induced Ca^{2+} release

(a) Different concentrations of carvedilol or DMSO were preincubated for 2 minutes with a subsequent addition of a sub-maximal concentration of cADPR (0.3μM) and the raw control response of cADPR was 72 ± 5 nM (n = 9). Summarized representative fluorimetric traces shown in (a) and data fit as a sigmoidal concentration-response shown in (b). Statistical significance was determined with a Dunnett's test in comparison to control response without carvedilol (b). Data are represented as mean ± SEM.

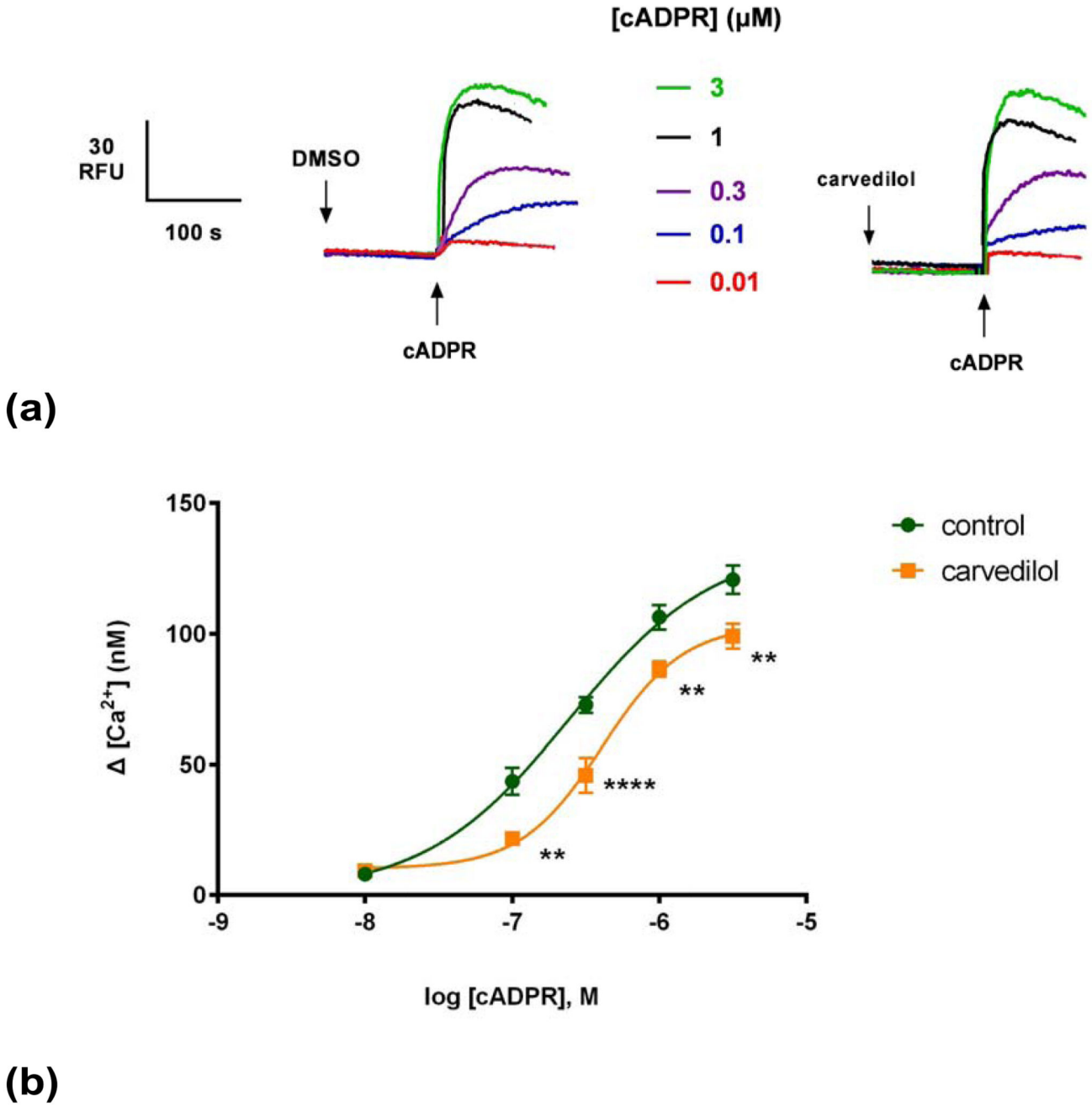

Fig. 2. Carvedilol inhibits cADPR-induced Ca^{2+} release

(a) Summarized representative fluorimetric traces. (b) DMSO or 30 μM of carvedilol were preincubated for 2 minutes before the addition of different cADPR concentrations. Statistical significance was determined using a Student's t test comparing control ($n = 9$) and carvedilol ($n = 9$) (b). Data were fit as a sigmoidal concentration-response (b). Data are represented as mean ± SEM.

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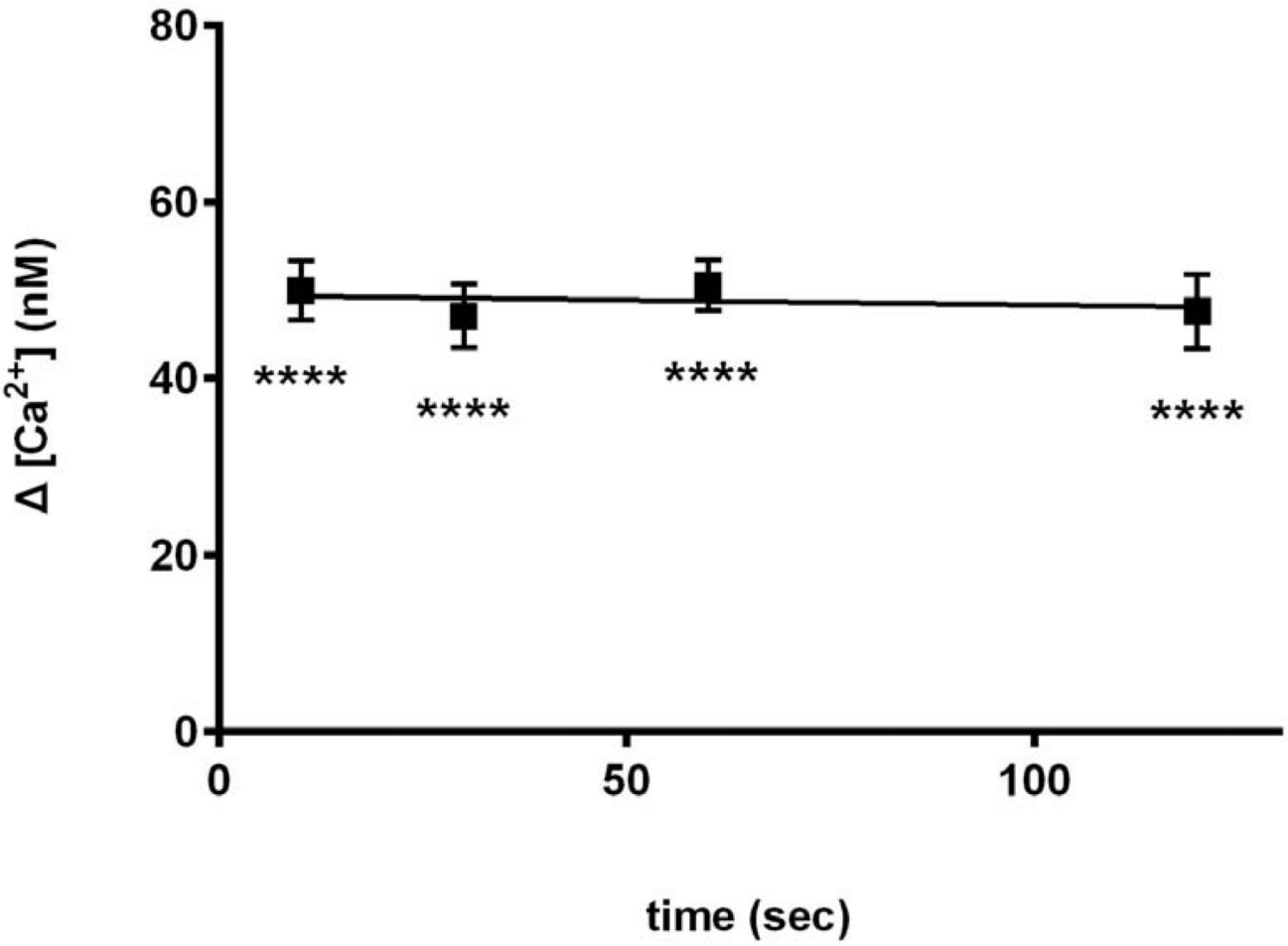

Fig. 3. Carvedilol suppresses cADPR-induced Ca^{2+} release rapidly

Effect of 30 μM carvedilol after preincubation with carvedilol for 10 s, 30 s, 60 s and 100 s is shown. Uncalibrated control Δ[Ca^{2+}] was 76 ±4 nM (n = 9). Statistical significance was determined using Dunnett's test versus 0 μM of carvedilol. Data are represented as mean ±SEM.

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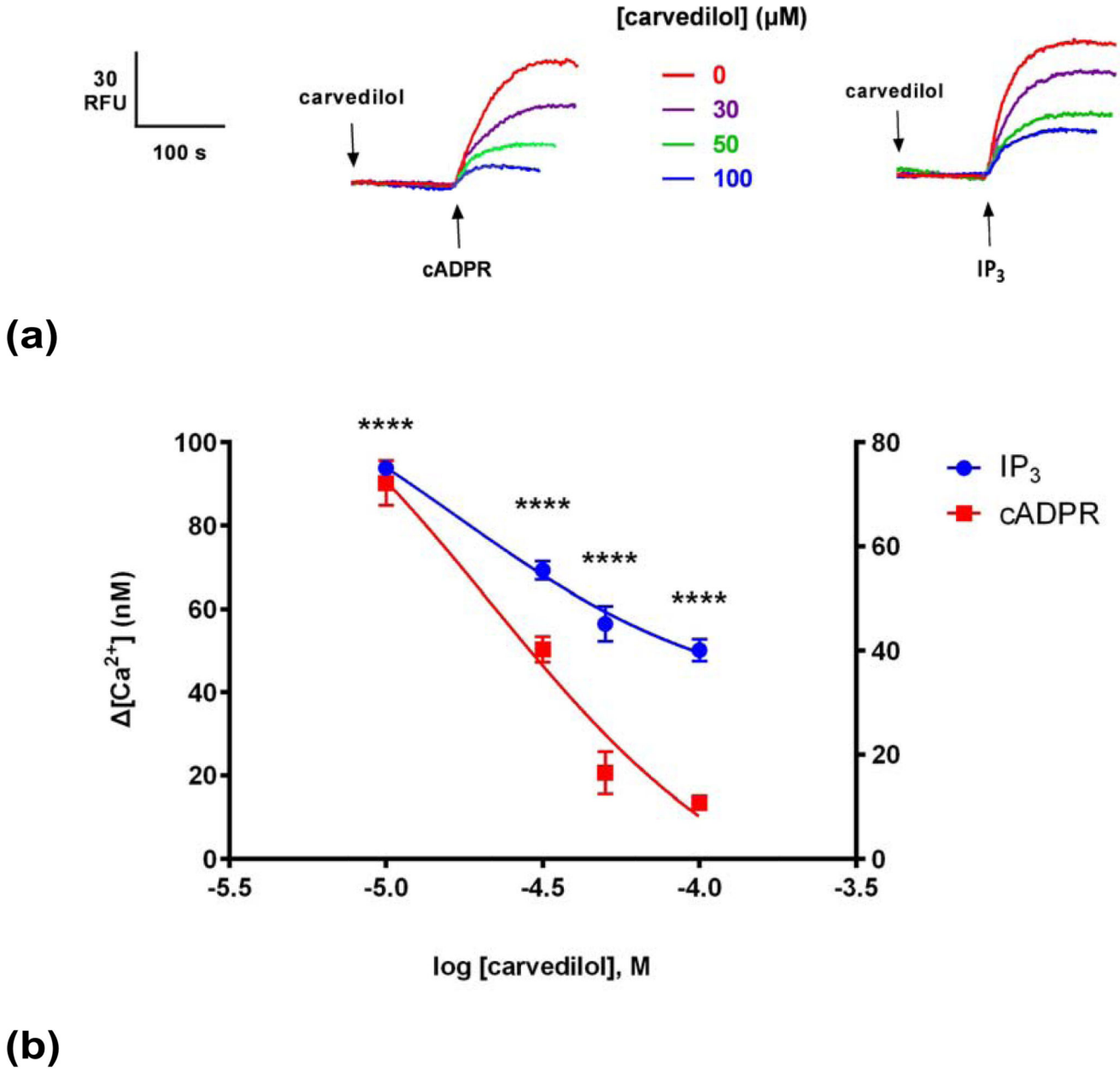

Fig. 4. Carvedilol inhibits IP3-induced Ca^{2+} release

(a) Different concentrations of carvedilol were preincubated for 2 minutes before the addition of sub-maximal concentrations of either IP_3 or cADPR (0.3 μM), which were determined by establishing dose-response curves. Raw control Δ[Ca^{2+}] with cADPR was 72 ±4 nM (n = 4–7) and with IP3 94 ±1 nM (n = 4–7).(b) Graph summarizing the results. Right Y axis applies for IP3, whereas left Y axis for cADPR. Statistical significance was determined using Student's t test and data were fit as a sigmoidal concentration-response. Data are represented as mean ±SEM.

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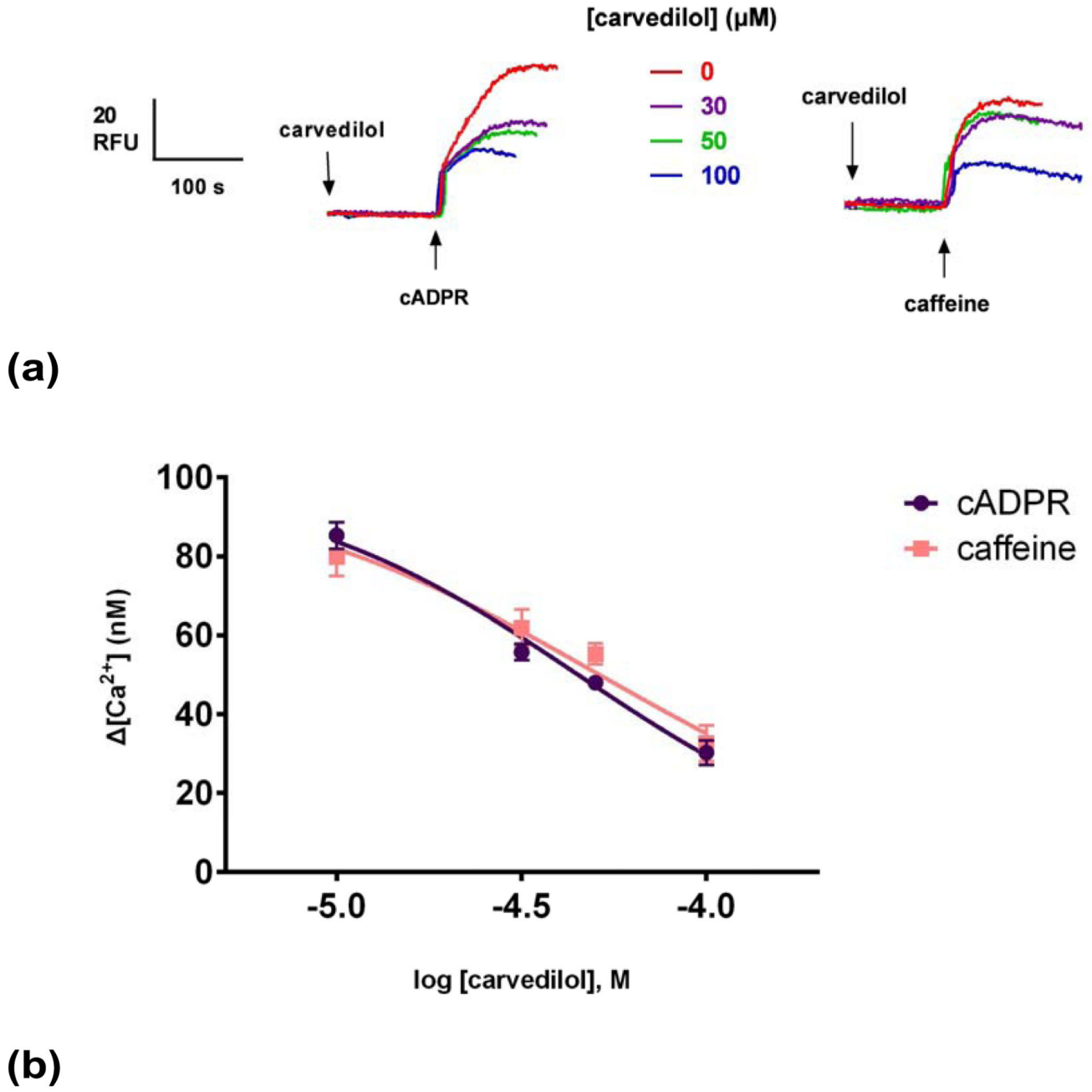

Fig. 5. Carvedilol suppresses caffeine-induced Ca^{2+} release

Different concentrations of carvedilol were preincubated for 2 minutes before the addition of sub-maximal concentrations of either caffeine (10 mM) or cADPR (0.3 μM). Raw control $\Delta[Ca^{2+}]$ with caffeine was 80 ±5 nM (n = 4–7) and with cADPR 85 ±3 (n = 4–7). Statistical significance (P>0.05) was determined using Student's t test and data were fit as a sigmoidal concentration-response. Summarized representative fluorimetric traces are shown in (a). Data are represented as mean ±SEM.

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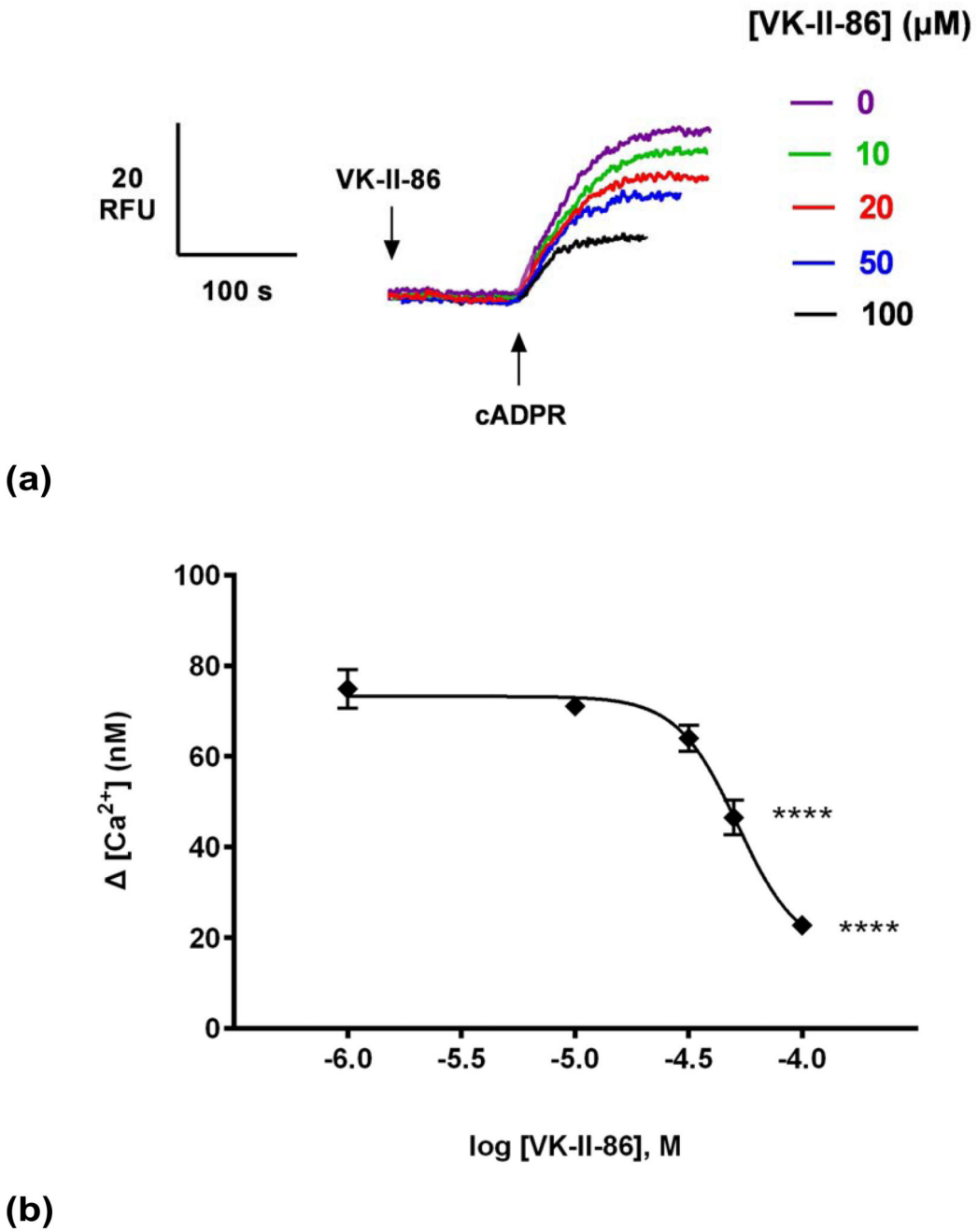

Fig. 6. VK-II-86 suppresses cADPR-induced Ca^{2+} release

(a) Different concentrations of VK-II-86 or DMSO were preincubated for 2 minutes with a subsequent addition of a sub-maximal concentration of cADPR (0.3 μM) and the raw control response of cADPR was 75 ±4 nM (n = 9). Summarized representative fluorimetric traces shown in (a) and data fit as a sigmoidal concentration-response shown in (b). Statistical significance was determined with a Dunnett's test in comparison to control response without carvedilol (b). Data are represented as mean ±SEM.

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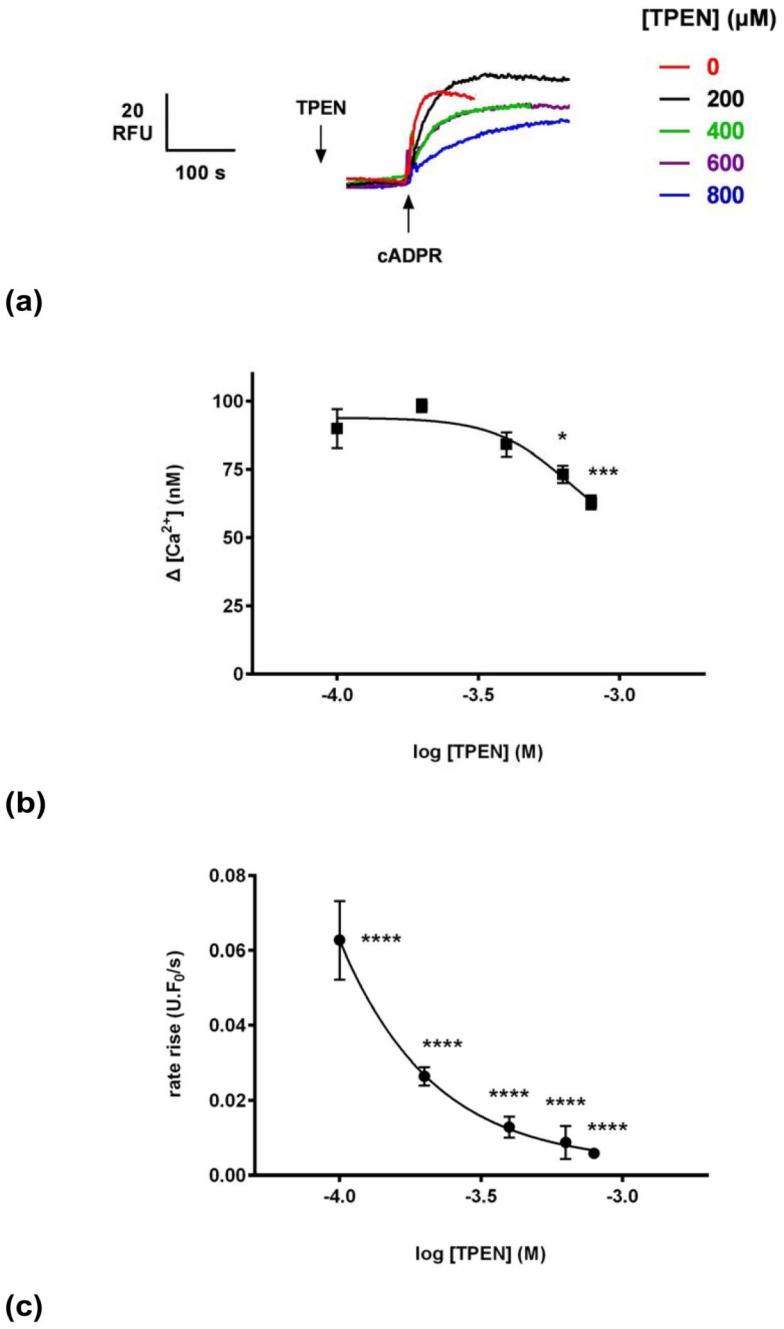

Fig. 7. TPEN inhibits cADPR-induced Ca^{2+} release

(a) Different concentrations of TPEN (or vehicle ethanol) were preincubated with egg homogenate for 2 minutes prior to the addition of 0.3 μM cADPR.

(b) TPEN reduces the magnitude of cADPR-evoked Ca^{2+} release.

(c) TPEN affects the kinetics of cADPR-evoked Ca^{2+} release. Fluorescence (in units (U)/s) was normalized to the resting fluorescence (F_0) to account for variability and therefore expressed as $U.F_0/s$. Statistical significance was determined using Student's t test and data were fit as a sigmoidal concentration-response. Data are represented as mean ±SEM.

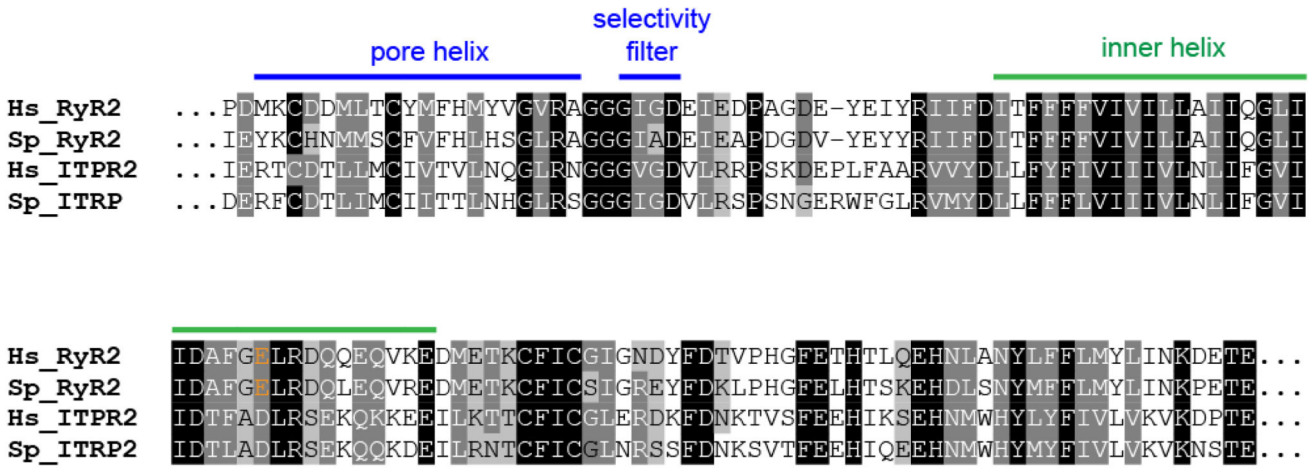

Fig. 8. Key amino acid residues for the proposed luminal Ca^{2+} sensor of RyRs and IP_3Rs are conserved in sea urchins

Alignment of amino acid sequences of the pore and inner helix region of RyR2 and IP3R2 from human (Hs) and sea urchin (Sp; *Strongylocentrotus purpuratus*). The acidic nature of residue E4872 (in orange) is conserved between RyR2 and IP3R2. Sequences (HsRyR2: Q92736; SpRyR2: XP_011670306; HsITPR2: Q14571; SpITPR2: XP_011682423) were aligned using Clustal Omega.