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cADPR stimulates SERCA activity in *Xenopus* oocytes

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ABSTRACT

The intracellular second messenger cyclic ADP-ribose (cADPR) induces Ca^{2+} release through the activation of ryanodine receptors (RyRs). Moreover, it has been suggested that cADPR may serve an additional role to modulate sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump activity, but studies have been complicated by concurrent actions on RyR. Here, we explore the actions of cADPR in *Xenopus* oocytes, which lack RyRs. We examined the effects of cADPR on the sequestration of cytosolic Ca^{2+} following Ca^{2+} transients evoked by photoreleased inositol 1,4,5-trisphosphate ($InsP_3$), and by Ca^{2+} influx through expressed nicotinic acetylcholine receptors (nAChR) in the oocytes membrane. In both cases the decay of the Ca^{2+} transients was accelerated by intracellular injection of a non-metabolizable analogue of cADPR, 3-Deaza-cADPR, and photorelease of cADPR from a caged precursor demonstrated that this action is rapid (a few s). The acceleration was abolished by pre-treatment with thapsigargin to block SERCA activity, and was inhibited by two specific antagonists of cADPR, 8-NH_2-cADPR and 8-br-cADPR. We conclude that cADPR serves to modulate Ca^{2+} sequestration by enhancing SERCA pump activity, in addition to its well-established action on RyRs to liberate Ca^{2+}.

1. Introduction

Diverse cellular functions are regulated by changes in cytosolic $[Ca^{2+}]$. In addition to Ca^{2+} influx across the plasma membrane, intracellular organelles including the endoplasmic/sarcoplasmic reticulum (ER/SR), Golgi apparatus, mitochondria and lysosome-related acidic compartments serve as Ca^{2+} sources [1–5]. Liberation of Ca^{2+} from these stores is regulated by intracellular Ca^{2+}-releasing messengers that act on Ca^{2+}-permeable receptor/channel molecules in the organelle membranes. One major pathway involves inositol 1,4,5-trisphosphate ($InsP_3$), which binds to $InsP_3$ receptor/channels ($InsP_3R$) in the endoplasmic reticulum (ER) membrane [6,7]. $InsP_3$-mediated Ca^{2+} signalling is a well-established system in many cell types, and has served as paradigm for discovery of other Ca^{2+}-mobilizing messengers [8–10]. A second major pathway involves ryanodine receptors (RyRs), which are abundantly expressed in ER/SR membranes. Both $InsP_3R$ and RyR channels are regulated by cytosolic Ca^{2+} itself, and are further modulated by enzymes such as PKA [11–13].

Following a Ca^{2+} signal, cytosolic $[Ca^{2+}]$ must ultimately be restored to its basal level. Canonical mechanisms involved in cytosolic Ca^{2+} removal are the plasma membrane Ca^{2+}-ATPase and Na^+/Ca^{2+}-exchanger, the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and the mitochondria Ca^{2+} uniporter [14]. The relative contributions of those Ca^{2+} removal mechanisms vary widely among cell types and with developmental stage [15,16].

The nucleotide, cADPR has been implicated as an additional Ca^{2+}-mobilizing messenger [9,17]. It is synthesized by ADPR-ribose cyclase enzymes, CD38 or CD157 in mammals [18,19], which are widely distributed, pointing to a ubiquitous role of cADPR. The ability of cADPR to mobilize cytosolic Ca^{2+} is distinct from that of $InsP_3$, but instead is thought to involve an action on RyRs in the ER membrane. The interaction of cADPR with RyRs to enhance Ca^{2+} liberation was initially demonstrated in sea urchin egg homogenates by showing the sensitivity of cADPR-mediated Ca^{2+} release to pharmacological inhibitors of RyRs [20], and has subsequently been studied extensively in cardiac muscle. Most studies concur that the primary target of cADPR is the RyR [21–28]. However, a report by Lukyanenko showing that Ca^{2+} uptake by cardiac microsomes is accelerated by cADPR led to the suggestion that the enhanced Ca^{2+} liberation through RyR activity may also arise indirectly because increased SR Ca^{2+} uptake leads to an elevated SR Ca^{2+} content [29,30]. This hypothesis consistent also with more recent studies [31] demonstrating a dual effect of cADPR in ventricular myocytes; that is, an initial, rapid effect to increase SR Ca^{2+} release by changing sensitivity of RyRs to cytosolic Ca^{2+} without changing SR Ca^{2+}

Abbreviations: cADPR, cyclic ADP-ribose; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; ER, endoplasmic reticulum; nAChR, nicotinic acetylcholine receptor; PKA, protein kinase A; RyR, ryanodine receptor.

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content or affecting L-type Ca^{2+} channels, followed by a slower enhancement of SR Ca^{2+} levels consistent with an increased rate of Ca^{2+} uptake.

Studies to confirm and elucidate how cADPR may modulate SR Ca^{2+} uptake are obviously complicated in cell types that display RyR-mediated Ca^{2+} liberation. In the present study, we thus address whether cADPR exerts a specific action on SERCA by using *Xenopus laevis* oocytes, which express $InsP_3$Rs but not RyRs [32]. Consistent with this, and with earlier reports [33], photorelease of cADPR failed to evoke detectable Ca^{2+} signals in oocytes. We then examined the effects of cADPR on the clearance of cytosolic Ca^{2+} transients evoked by photoreleased $InsP_3$ and by influx through expressed plasmalemmal nicotinic acetylcholine receptors (nAChR) in response to hyperpolarizing voltage-clamped pulses. Both photoreleased cADPR and intracellular loading of a non-metabolizable cADPR analogue, 3-Deaza-cADPR [34], accelerated the decay of these cytosolic Ca^{2+} transients. Moreover, this acceleration was abolished by the specific SERCA inhibitor thapsigargin [35], and was antagonised by the cADPR inhibitors 8-NH_2-cADPR and 8-Br-cADPR [36]. We thus conclude that cADPR may play a physiological role in promoting SERCA activity.

2. Methods

2.1. Oocyte preparation

X. laevis (purchased from Nasco International, Fort Atkinson, WI, USA) were sacrificed according to the protocols approved by the UC Irvine Institutional Animal Care and Use committee, and stage V–VI oocytes were isolated after removing ovaries. Oocytes were treated with collagenase (1 mg/ml of collagenase type A1 for 30 min) to remove enveloping cell layers and were stored in modified Barth's solution (mM: NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.82; $Ca(NO_3)_2$, 0.33; $CaCl_2$, 0.41; HEPES, 5; gentamicin, 1 mg/ml; pH 7.4) for 1–5 days before use.

2.2. Expression of nAChRs

Plasmids containing cDNA clones coding for the muscle nicotinic receptor α, β, γ, and δ subunits were linearized and transcribed in vitro, and corresponding cRNAs (molar ratio 2:1:1:1) were mixed to a final concentration of 0.1–1 mg/ml and injected (50 nl) into oocytes. Oocytes were maintained at 16 °C for 1–3 days to express nACh-Rs in the plasma membrane. Expression level was monitored using a voltage clamp to measure current in response to 100–500 nM ACh: oocytes showing currents >1 μA at −80 mV were selected for experiments.

2.3. Microinjection of oocytes

Intracellular microinjections were performed using a Drummond microinjector in Ca^{2+}-free Barth's solution. Final intracellular concentrations given below were calculated assuming a cytosolic volume of 1 μl. Oocytes were loaded with combinations of following: fluo 4 dextran, high affinity, 40 μM; Oregon Green 488 BAPTA-5N (OGBTA-5N), 40 μM; $P_{4(5)}$-(1-(2-nitrophenyl)ethyl)ester, Tris (triethylammonium) salt (caged-$InsP_3$), 8 μM; cyclic adenosine 5′-diphosphate ribose, 1-(1-(2-nitrophenyl)ethyl ester) (caged-cADPR), 16 μM; cyclic 3-Deaza-adenosine 5′-diphosphate ribose (3-Deaza-cADPR), 0.8 μM; 8-amino-cyclic adenosine 5′-diphosphate ribose (8-NH_2-cADPR), 16 μM; 8-bromo-cyclic adenosine diphosphate ribose (8-Br-cADPR), 80 μM. Oocytes were left for 15–30 min after injections to allow agents to diffuse throughout the cell before use.

2.4. Confocal laser scanning microscopy

Confocal linescan Ca^{2+} images were obtained as described previously [37], employing a custom-built confocal scanner interfaced to an Olympus IX70 inverted microscope [38]. Fluorescence excitation was provided by the 488 nm line of an argon ion laser, with the laser spot focused by a 40× oil immersion objective (NA 1.35) and scanned along a 50 μm line. Emitted fluorescence was detected at wavelengths >510 nm through a confocal pinhole, providing lateral and axial resolutions of about 0.3 and 0.8 μm, respectively. Images were collected using custom-written image acquisition software (Labview). Recordings were made at 16–18 °C, imaging at the level of the pigment granules in the animal hemisphere of oocytes bathed in normal Ringer solution (mM: $NaCl_2$, 120; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; pH 7.4). $InsP_3$ was uniformly photoreleased throughout a 100 μm spot surrounding the image scan line [39], and the relative concentration of $InsP_3$ was controlled by using an electronic shutter to vary flash duration and/or neutral density (ND) filters. Intervals of 2 min were allowed between recordings. Fluorescence signals are expressed as ratios ($\Delta F/F_0$) of the fluorescence (F) at each pixel relative to the mean resting fluorescence (F_0) at that pixel prior to stimulation. Custom routines written in the IDL programming environment (Research Systems, Boulder, CO, USA) were used for image processing and measurements were exported to Microcal Origin version 6.0 (OriginLab, Northamptom, MA, USA) for analysis and graphing.

2.5. Reagents

Fluo 4 dextran, high affinity (K_d: ~350 nM), caged-$InsP_3$, caged-cADPR and 8-NH_2-cADPRwere purchased from Invitrogen (Carlsbad, CA, USA). 3-Deaza-cADPR and collagenase type A were from Sigma–Aldrich (St. Louis, MO, USA), and 8-Br-cADPR (IC_{50}: ~1 μM) was from BIOMOL International, L.P. (Plymouth Meeting, PA, USA).

3. Results

3.1. cADPR does not mobilize Ca^{2+} in oocytes

We first confirmed previous findings that cADPR fails to evoke Ca^{2+} liberation in *Xenopus* oocytes [33]. Oocytes were loaded with the high affinity (K_d = 345 nM) Ca^{2+} indicator fluo-4 dextran (40 μM final intracellular concentration) and caged-cADPR (16 μM final intracellular concentration). No responses were detected upon even very strong stimuli (repeated sequences of 200 ms flashes at maximal UV intensity; 3 regions tested in each of 3 oocytes); whereas weaker photolysis flashes evoked large responses in oocytes loaded with caged-$InsP_3$ (8 μM final intracellular concentration) (e.g. Fig. 1A). Moreover, no change in basal Ca^{2+}-dependent fluorescence was seen after injecting oocytes with the non-metabolizable analogue 3-Deaza-cADPR to a final intracellular concentration of 0.8 μM (control: 4.3 ± 1.0 fluorescence unit; 3-Deaza-cADPR: 4.3 ± 0.4 fluorescence unit, n = 10, p > 0.05).

3.2. cADPR accelerates the decay of $InsP_3$-evoked Ca^{2+} transients

We then explored possible actions of cADPR independent of Ca^{2+} mobilization through RyRs by examining the effects of 3-Deaza-cADPR on global Ca^{2+} signals generated by photorelease of $InsP_3$ from a caged precursor. Fig. 1A shows representative confocal linescan images of global Ca^{2+} transients evoked by relatively strong photorelease of $InsP_3$ in a control oocyte (upper panel) and in an oocyte previously loaded with 3-Deaza-cADPR (lower). To compare kinetics in the face of cell-to-cell variability we normalized responses to individual peak amplitudes (Fig. 1B), and measured

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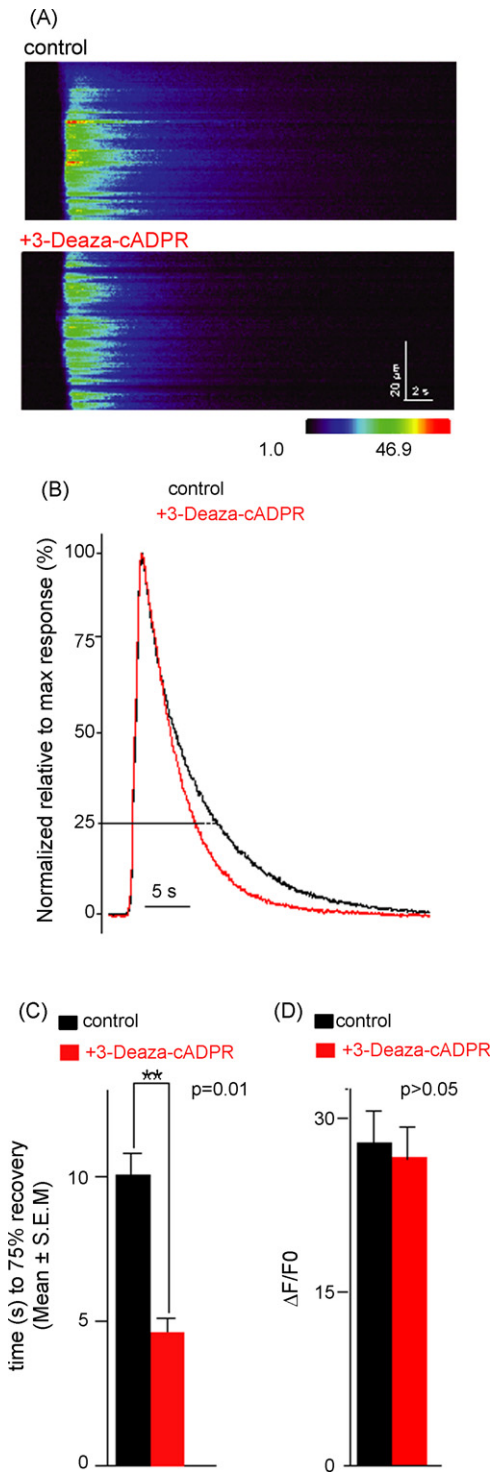

Fig. 1. 3-Deaza-cADPR accelerates the decay of global Ca^{2+} signals generated by photoreleased $InsP_3$. (A) Representative confocal line scan images illustrating fluo-4 dextran fluorescence signals evoked by photoreleased $InsP_3$ in a control oocyte (upper panel) and in an oocyte pre-loaded with 3-Deaza-cADPR to a final cytosolic concentration of ~0.8 μM (lower panel). In both cases the durations of the photolysis flashes was about double the threshold required to evoke a detectable Ca^{2+} signal. (B) Fluorescence profiles from (A) averaged across the scan line, after normalizing peak amplitudes to 100%. (C) Mean decay times (75% recovery from peak to baseline) of fluorescence signals in control oocytes (black; 10.0 ± 0.8 s n = 6 oocytes), and 3-Deaza-cADPR-loaded oocytes (red; 4.6 ± 0.5 s, n = 10, p = 0.01). (D) Mean peak fluorescence signals are not significantly different (p > 0.05) between control ($\Delta F/F_0$ = 27.8 ± 3.0, n = 6) and 3-Deaza-cADPR-loaded oocytes ($\Delta F/F_0$ = 26.6 ± 2.8, n = 10). (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

times to 75% recovery from peak to baseline. The mean recovery time was accelerated about 2-fold by 3-Deaza-cADPR (Fig. 1C: control, 10.0 ± 0.8 s, n = 6 oocytes; 3-Deaza-cADPR, 4.6 ± 0.5 s, n = 10, p < 0.01), whereas there was no significant effect of 3-Deaza-cADPR on peak amplitudes (Fig. 1D).

3.3. *Thapsigargin inhibits the action of cADPR to accelerate the decay of $InsP_3$-evoked Ca^{2+} signals*

To address whether cADPR affects Ca^{2+} sequestration by targeting SERCA activity we used thapsigargin as a specific inhibitor of the SERCA pump [35]. Global Ca^{2+} signals were evoked by flash photorelease of $InsP_3$ as before, and paired measurements of decay rates were obtained in the same oocytes before and 15 min after addition of 20 μM thapsigargin to the bathing solution. Surprisingly, thapsigargin caused only a slight slowing of Ca^{2+} decay in control oocytes (Fig. 2A: 75% recovery before thapsigargin 10.1 ± 0.8 s; after thapsigargin 11.3 ± 1.3 s, n = 6, p > 0.05), suggesting that SERCA activity plays only a minor role in the decay of $InsP_3$-evoked Ca^{2+} signals under basal conditions. On the other

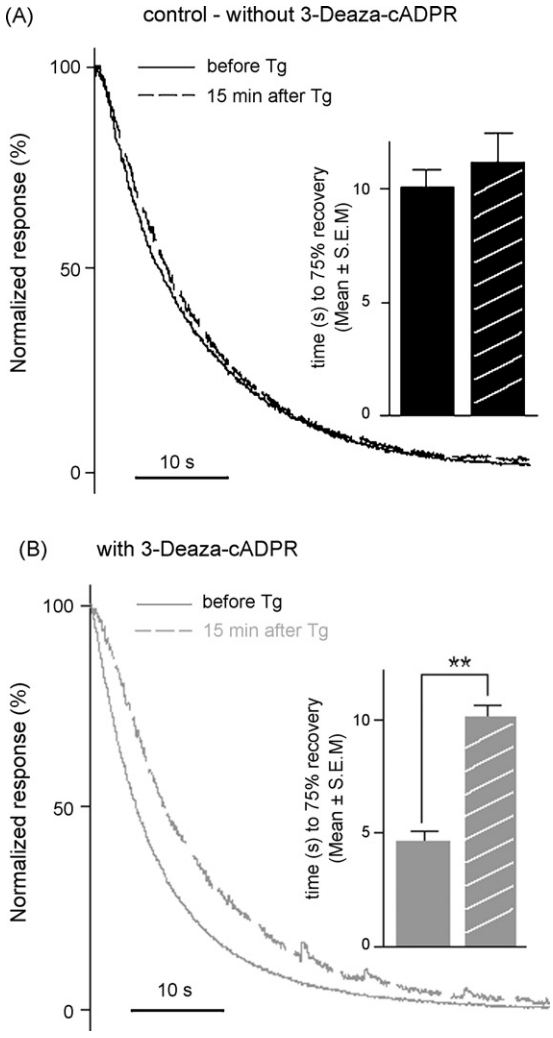

Fig. 2. Thapsigargin antagonises the effect of 3-Deaza-cADPR on accelerating the decay of Ca^{2+} signals evoked by photoreleased $InsP_3$. (A) Corresponding decay times in control oocytes (without 3-Deaza-cADPR) before (solid black, 10.1 ± 0.8 s) and 15 min after application of 20 μM thapsigargin (broken black, 11.3 ± 1.3 s, n = 6, p > 0.05). (B)Thapsigargin slowed the decay of $InsP_3$-evoked Ca^{2+} signals in oocytes pre-loaded with 3-Deaza-cADPR (solid grey, before thapsigargin, 4.7 ± 0.5 s; broken grey, 15 min after thapsigargin, 10.2 ± 0.5 s, n = 10, p = 0.01).

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hand, oocytes loaded with 3-Deaza-cADPR showed a more rapid Ca^{2+} decay (4.7 ± 0.5 s, n = 10 vs. 10.1 ± 0.8 s, n = 6 in control oocytes; p = 0.01). Importantly, this was markedly slowed following thapsigargin treatment, returning close to the value in control cells without cADPR (Fig. 2B: 75% recovery after thapsigargin 10.2 ± 0.5 s, n = 10, p = 0.01).

3.4. cADPR accelerates clearance of Ca^{2+} entering through expressed plasmalemmal nAChRs

The results in Fig. 2 suggest that cADPR likely accelerates cytosolic Ca^{2+} clearance by promoting SERCA pump activity. However, interpretation of the decay rate of $InsP_3$-evoked Ca^{2+} signals is complicated because this reflects both the rates of sequestration mechanisms that remove Ca^{2+} ions from the cytosol, and the rate at which Ca^{2+} flux into the cytosol through $InsP_3R$ terminates [40]. To address whether the action of cADPR on Ca^{2+} sequestration is independent of activity of $InsP_3Rs$, we expressed Ca^{2+}-permeable nicotinic acetylcholine receptors (nAChRs) in the oocyte membrane to serve as a 'Ca^{2+} switch' so as to precisely control the kinetics of cytosolic Ca^{2+} elevations [41,42]. Acetylcholine was maintained at a low, non-desensitizing concentration in the bathing solution. Oocytes were voltage clamped at 0 mV to minimize the electrochemical gradient for Ca^{2+} influx at rest and pulsed to −120 mV to evoke Ca^{2+} transients. Fig. 3A shows representative line scan images in response to hyperpolarizing steps with durations of 1.0 and 3.0 s, and corresponding measurements of fluorescence ratio changes averaged across the scan line are shown in Fig. 3B. The decay of the fluorescence signal following termination of Ca^{2+} influx upon stepping back to 0 mV was appreciably slower with the 3 s pulse than with the 1 s pulse. A likely explanation for this difference is that decay of [Ca^{2+}] after a brief influx is dominated by passive diffusion of Ca^{2+} ions into the enormous interior volume of the oocyte, whereas this process is slowed by accumulation of Ca^{2+} during longer pulses so that other active sequestration mechanisms assume greater prominence.

We next compared the decay of normalized signals in control oocytes (Fig. 3C, black traces) with that in oocytes pre-loaded with 0.8 μM 3-Deaza-cADPR (grey traces). An acceleration was evident following both 1.0 and 3.0 s hyperpolarizing pulses, and mean data are summarized by the inset bar graphs in Fig. 3C (1.0 s hyperpolarizing pulse: control, 1.7 ± 0.2 s, n = 4, and 3-Deaza-cADPR, 0.9 ± 0.3 s, n = 3, p = 0.03; 3.0 s hyperpolarizing pulse: control, 3.7 ± 0.2, n = 25, and 3-Deaza-cADPR, 2.9 ± 0.2 s, n = 36, p < 0.01).

3.5. Rapid action of cADPR on accelerating Ca^{2+} uptake

The experiments described above utilized a non-metabolizable cADPR analogue pre-loaded into the oocyte several minutes before recording. To then determine how rapidly cADPR may act on Ca^{2+} sequestration we used photolysis of caged-cADPR to elevate cytosolic [cADPR] shortly before inducing Ca^{2+} influx through nAChRs (Fig. 4A). Control experiments showed that exposure to UV light in oocytes not loaded with caged-cADPR produced no artifactual change in Ca^{2+} decay kinetics following a hyperpolarizing pulse to induce Ca^{2+} influx (Fig. 4B: 3.6 ± 0.4 s without UV in solid black; 3.5 ± 0.3 s with UV in broken black; p > 0.05). In contrast, sustained exposure to photolysis light beginning 5.0 s before the 3.0 s hyperpolarizing pulse resulted in a small but statistically significant acceleration of decay in oocytes loaded with 16 μM caged-cADPR (Fig. 4C: 75% recovery times 3.2 ± 0.1 s without UV exposure grey trace, 2.7 ± 0.1 s with exposure, broken trace, n = 10, p < 0.05).

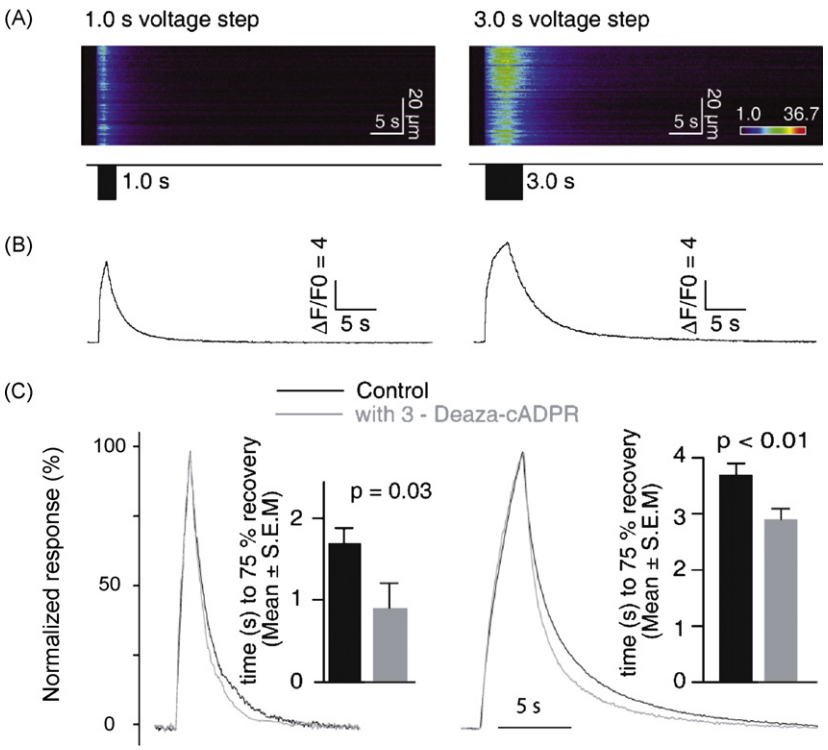

Fig. 3. 3-Deaza-cADPR accelerates the decay of Ca^{2+} signals evoked by Ca^{2+} influx through nAChRs expressed in the plasma membrane. (A) Linescan images illustrating Ca^{2+} transients evoked by stepping the membrane potential from 0 to −120 mV for 1.0 s (left) or 3.0 s (right) to increase the electrochemical driving force for influx of extracellular Ca^{2+} through nAChR activated by 100–500 nM ACh in the bathing solution. (B) Corresponding fluorescence profiles averaged across the scan line. (C) Comparison of averaged, normalized fluorescence profiles from control oocytes (black) and oocytes pre-loaded with 3-Deaza-cADPR (grey) for hyperpolarizing pulses of 1.0 s (left) and 3.0 s (right) duration. Inset bar graphs show mean values of 75% decay times.

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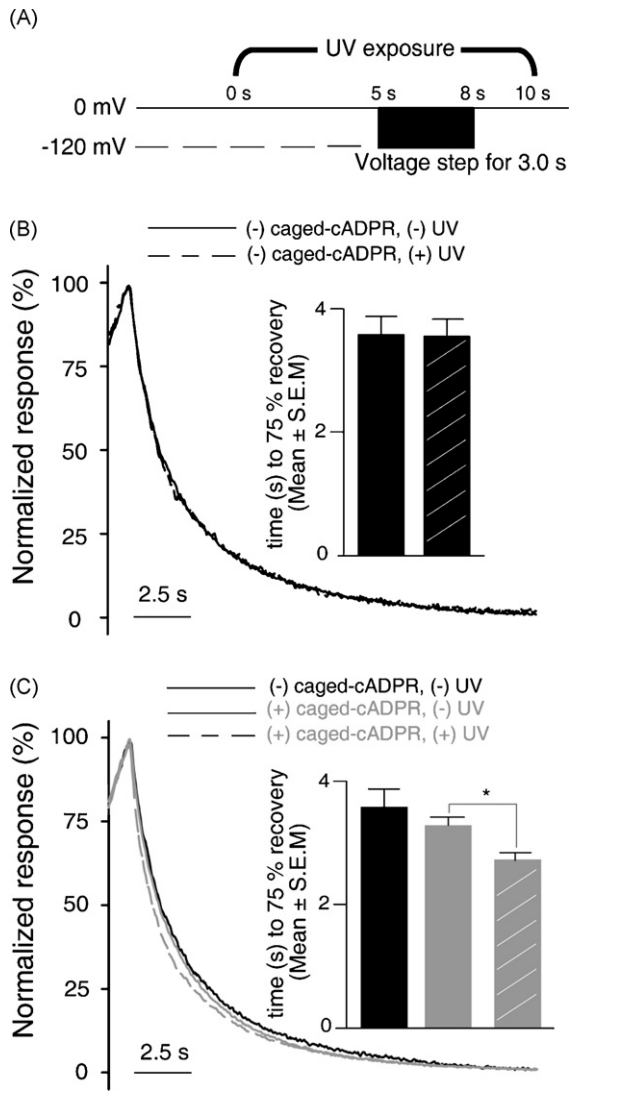

Fig. 4. Photorelease of cADPR rapidly induces an acceleration of Ca^{2+} decay. (A) Diagram illustrating the experimental protocol. (B) Control experiment, comparing Ca^{2+} decay kinetics with and without UV exposure in oocytes not loaded with 3-Deaza-cADPR. Traces show averaged, normalized fluorescence profiles, beginning 1.0 s before the end of the hyperpolarizing pulse. Inset bar graphs show paired measurements of mean decay times to fall to 75% of peak in 4 oocytes (without UV in solid black and with UV in broken black). (C) Photorelease of cADPR accelerates Ca^{2+} decay. Traces and bar graphs compare decay kinetics in oocytes not loaded with caged cADPR and not exposed to UV (black), loaded with caged cADPR but not exposed to UV (solid grey) and loaded with caged cADPR and exposed to UV (broken grey).

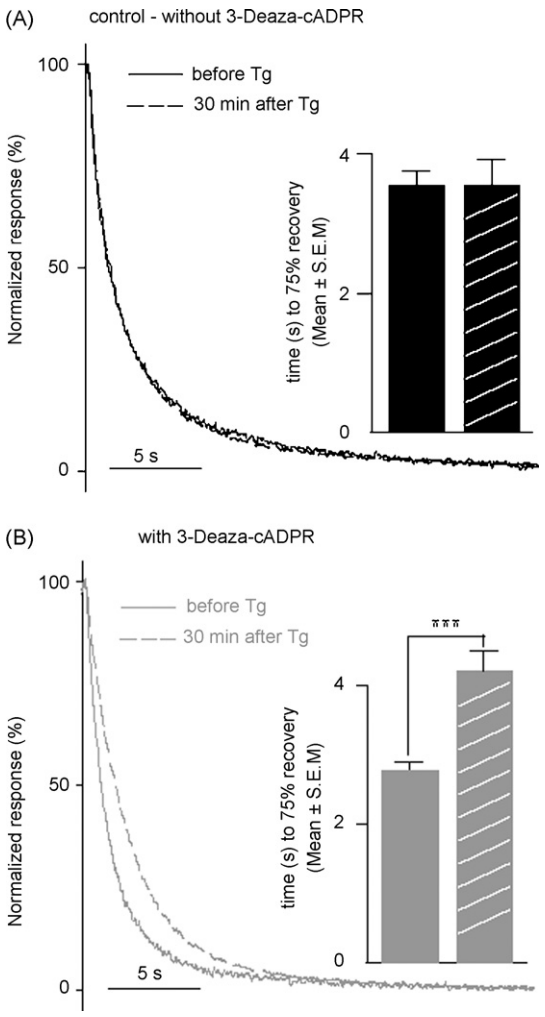

Fig. 5. Thapsigargin antagonizes the effect of 3-Deaza-cADPR on accelerating the decay of Ca^{2+} following influx through nAChRs. Data were obtained following the protocol in Fig. 3, using 3 s hyperpolarizing pulses. (A) Representative examples of Ca^{2+} decay in a control oocytes (without 3-Deaza-cADPR) before (black trace) and after (broken trace) treatment with 20 μM thapsigargin for 30 min. Inset histogram shows mean values of 75% decay time (before thapsigargin: 3.7 ± 0.2 s, n = 25; after thapsigargin: 3.7 ± 0.4 s, n = 5, p > 0.05). (B) Corresponding traces and measurements showing that thapsigargin reversed the acceleration of Ca^{2+} decay in oocytes injected with 3-Deaza-cADPR (before thapsigargin: 2.9 ± 0.1, n = 36; after thapsigargin: 4.4 ± 0.3, n = 10, p < 0.01).

3.6. Thapsigargin blocks the cADPR-mediated acceleration of Ca^{2+} decay following influx through nAChRs

To confirm whether the effect of cADPR on speeding the clearance of Ca^{2+} that had entered the cytosol through nAChR is mediated via actions on SERCA, we again applied 20 μM thapsigargin to block SERCA activity. In control oocytes (without 3-Deaza-cADPR), incubation with thapsigargin for 30 min resulted in almost no change in decay of the Ca^{2+} transient following influx evoked by 3.0 s hyperpolarizing pulses (Fig. 5A: 75% decay time before thapsigargin, 3.7 ± 0.2 s, n = 25 oocytes; with thapsigargin 3.7 ± 0.4, n = 5 oocytes, p > 0.05); concordant with the similar lack of effect on decay of $InsP_3$-evoked Ca^{2+} transients (see Fig. 2A). On the other hand, the accelerated decay seen in oocytes loaded with 3-Deaza-cADPR was substantially slowed by thapsigargin (Fig. 5B: 75% recovery time before thapsigargin 2.9 ± 0.1 s, n = 36; with thapsigargin 4.4 ± 0.3 s, n = 10, p < 0.01).

3.7. cADPR inhibitors antagonise the action of 3-Deaza-cADPR on Ca^{2+} clearance

8-NH_2-cADPR and 8-br-cADPR are competitive antagonists of cADPR modulation of RyR-mediated Ca^{2+} signalling in many cell types, with respective IC_{50} values of 9 nM and 1 μM in inhibiting Ca^{2+} signals induced by cADPR in sea urchin egg homogenate [36]. We thus examined whether they would similarly antagonise the action of cADPR on accelerating cytosolic Ca^{2+} clearance following influx through nAChRs. Comparison of decay rates in oocytes injected with 3-Deaza-cADPR alone (75% recovery time 2.9 ± 0.2 s, n = 36) with those in oocytes loaded also with 8-NH_2-cADPR (16 μM final intracellular concentration) showed a strong antagonism (Fig. 6A: 75% recovery time, 3.6 ± 0.5 s, n = 10, p = 0.02). A lesser slowing was also apparent in oocytes loaded with 80 μM 8-br-cADPR (Fig. 6B: 75% recovery time 3.3 ± 0.3 s, n = 10, p = 0.03).

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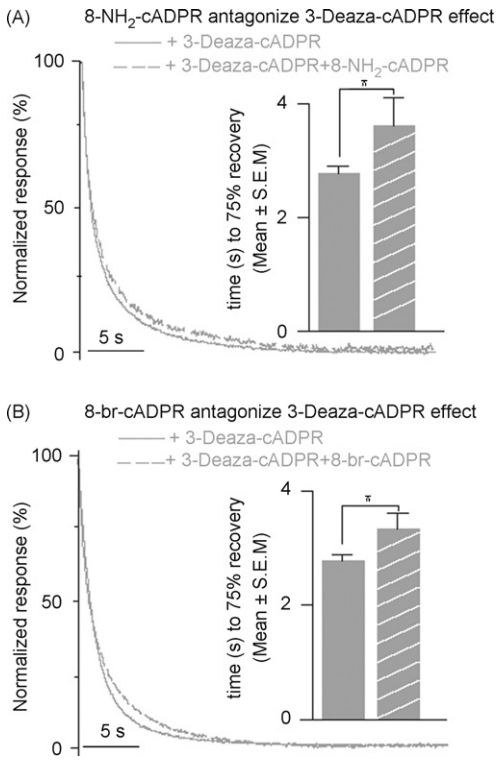

Fig. 6. Competitive inhibitors of cADPR action antagonize the effect of 3-Deaza-cADPR on accelerating Ca^{2+} clearance. (A) Traces and bar graphs compare the kinetics of Ca^{2+} decay measured as in Fig. 3 following influx through nAChR in oocytes injected with 3-Deaza-cADPR alone (solid grey trace, mean time to decay to 75% from peak 2.9 ± 0.2 s, $n = 36$) and together with 8-NH_2-cADPR (broken grey trace, 3.6 ± 0.5 s, $n = 10$, $p < 0.05$). (B) Corresponding action of 8-Br-cADPR (decay to 75% of peak 3.3 ± 0.3 s, $n = 10$, $p < 0.05$).

4. Discussion

This study provides evidence for a distinct role of cADPR in the control of intracellular Ca^{2+} homeostasis via sequestration by SERCA pumps. Our main findings are that; (i) cytosolic calcium clearance is accelerated by cADPR, an action that is inhibited by the specific antagonists 8-NH_2-cADPR and 8-Br-cADPR, (ii) photorelease of cADPR from a caged precursor promotes acceleration of calcium clearance, and (iii) the effect of cADPR is abolished by thapsigargin, a specific blocker for SERCA pumps. Although previous reports had suggested that cADPR may modulate Ca^{2+} sequestration by SERCA [29,30], definitive studies were hampered by its well-established action to sensitize Ca^{2+} liberation through RyR. We thus investigated the actions of cADPR in *Xenopus* oocytes, which lack RyRs [32] and do not show Ca^{2+} signals in response to cADPR [33].

The most direct evidence comes from experiments utilizing Ca^{2+}-permeable nACh-R channels expressed in the oocyte membrane as a Ca^{2+} 'switch', whereby Ca^{2+} influx could be abruptly attenuated by stepping the membrane potential to more positive voltages such that the subsequent decay of intracellular Ca^{2+} fluorescence signal exclusively reflects Ca^{2+} removal from the cytosol. Oocytes pre-loaded with the non-metabolizable cADPR analogue 3-Deaza-cADPR showed a significantly acceleration in decay. This effect of 3-Deaza-cADPR was blocked by thapsigargin, indicating a specific action on SERCA.

Although the extent of the acceleration of Ca^{2+} clearance by cADPR appears relatively modest (<2-fold; Fig. 3), it must be remembered that SERCA is only one of several mechanisms (including sequestration by mitochondria; plasma membrane pumps and exchangers) removing Ca^{2+} from the cytosol. In particular, the enormous volume of the *Xenopus* oocyte makes passive diffusion into the interior of the cell a major factor contributing to the decline in cytosolic free [Ca^{2+}] adjacent to the plasmalemma [43]. Indeed, application of thapsigargin to control oocytes caused little detectable change in decay rate, suggesting that SERCA plays only a minor role in Ca^{2+} removal when measured in this way under basal conditions. Thus, the thapsigargin-sensitive speeding of decay induced by 3-Deaza-cADPR implies a much stronger regulatory action on SERCA than is immediately apparent from our records, and its effect on Ca^{2+} dynamics would be more prominent in cells of 'normal' size.

The decay of global $InsP_3$-evoked Ca^{2+} signals was accelerated more prominently by cADPR than was the clearance of Ca^{2+} entering across the plasma membrane, suggesting an action on the termination of Ca^{2+} liberation through $InsP_3R$ in addition to its effects on cytosolic Ca^{2+} clearance. cADPR has been shown to interact directly with the $InsP_3R$ (in particular the type 1 $InsP_3R$ present in *Xenopus* oocytes) [32], but an alternative explanation may be that $InsP_3R$ function is indirectly modulated by elevated [Ca^{2+}] in the ER lumen resulting from accelerated SERCA activity [44].

The mechanism by which cADPR exerts its action on SERCA remains unclear. Observation that photorelease of cADPR causes a small but significant acceleration of Ca^{2+} sequestration within <8 s would be consistent with a direct molecular interaction, but is not so rapid as to rule out a pathway involving intermediate proteins that may interact with SERCA after binding cADPR. In this context it is, however intriguing that unidentified cADPR-binding proteins apparently distinct from the RyRs have similar molecular weights (100 and 140 kDa) to isoforms of SERCA [45].

Modulation of SERCA activity has been intensively studied in the heart, where Ca^{2+} re-uptake into the SR is the predominant mechanism of sequestration from the cytosol [15]. SERCA activity in the heart is known to be enhanced by phosphorylation of phospholamban by cAMP-dependent protein kinase (PKA) [15]. Interestingly it has been indicated that an SR-associated ADP-ribose cyclase in the guinea-pig heart can be activated by PKA-mediated phosphorylation [46,47]. Moreover, in hydroid [46] it has been reported that PKA-mediated phosphorylation modulates ADP-ribose cyclase activity, adding further weight to the notion that cADPR may serve as a parallel pathway for SERCA regulation. Our results were obtained in oocytes which express type 2b and 3 isoforms of SERCA rather than the type 2a isoform in cardiac tissue. The observation that cADPR enhances pump rate by cardiac microsomes [29] suggests that this action is conserved across SERCA isoforms.

In summary, ample evidence indicates that a major role of cADPR is to modulate or directly activate RyRs in those tissues where RyRs are expressed [9]. We show that cADPR also modulates Ca^{2+} clearance in *Xenopus* oocytes where RyRs are not present. Thus, cADPR plays dual, apparently opposing roles in modulating the 'ins and outs' of Ca^{2+} signalling, by both potentiating Ca^{2+} liberation through RyR and accelerating its subsequent sequestration by SERCA. Most studies of cellular Ca^{2+} signalling have tended to emphasize the fast dynamic release of Ca^{2+} into the cytosol through ion channels as the predominant factor shaping cytosolic Ca^{2+} signals. However, there is a growing appreciation that Ca^{2+} clearance mechanisms such as SERCA are also an important site of modulation [48,49], and that disruptions in their physiological regulation may be implicated in diseases as diverse as heart failure [48–50] and Alzheimer's [51].

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