RESEARCH COMMUNICATION

Effects of photoreleased cADP-ribose on calcium transients and calcium sparks in myocytes isolated from guinea-pig and rat ventricle

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Actions of photoreleased cADP-ribose (cADPR), a novel regulator of calcium-induced calcium release (CICR) from ryanodine-sensitive stores, were investigated in cardiac myocytes. Photoreleased cADPR caused an increase in the magnitude of whole-cell calcium transients studied in mammalian cardiac ventricular myocytes (both guinea-pig and rat) using confocal microscopy). Approx. 15 s was required following photorelease of cADPR for the development of its maximal effect. Photoreleased cADPR also increased the frequency of calcium 'sparks', which are thought to be elementary events which make up the whole-cell calcium transient, and were studied in rat myocytes, but had little or no effect on spark characteristics (amplitude, rise time, decay time and distance to half amplitude). The potentiating effects of photoreleased cADPR on both whole-cell transients and the frequency of calcium sparks were prevented by cytosolic application of the antagonist 8-amino-cADPR

(5 μ M). These experiments, therefore, provide the first evidence in any cell type for an effect of cADPR on calcium sparks, and are the first to show the actions of photoreleased cADPR on whole-cell calcium transients in mammalian cells. The observations are consistent with the effects of cADPR in enhancing the calcium sensitivity of CICR from the sarcoplasmic reticulum in cardiac ventricular myocytes, leading to an increase in the probability of occurrence of calcium sparks and to an increase in whole-cell calcium transients. The slow time-course for development of the full effect on whole-cell calcium transients might be taken to indicate that the influence of cADPR on CICR may involve complex molecular interactions rather than a simple direct action of cADPR on the ryanodine-receptor channels.

Key words: excitation–contraction coupling, ryanodine receptor, sarcoplasmic reticulum.

INTRODUCTION

Actions of cADP-ribose (cADPR), both to release calcium from ryanodine-sensitive stores and to increase the calcium sensitivity of calcium-induced calcium release (CICR), have been described in a wide variety of tissues (see [1,2] for reviews), but effects of cADPR in intact cardiac myocytes have only recently been observed. Early studies with sarcoplasmic reticulum preparations [3] reported effects, whereas other studies did not detect effects under the conditions of the experiments [4], or observe changes which would be expected to be antagonized by physiological levels of ATP [5]. In early experiments on whole cardiac cells, effects of photolysis of caged cADPR [6] on calcium transients were not detected at room temperature [7], but more recent work has uncovered a marked temperature-dependence for the actions of cADPR in cardiac muscle [8]. These experiments showed that cADPR applied to the cytosol caused increases in calcium transients and contractions in guinea-pig ventricular cells at 36 °C. The actions were suppressed by ryanodine and thapsigargin, which interfere with the function of the sarcoplasmic reticulum, and were also antagonized by 8-amino-cADPR or 8-Br-cADPR. The observations were interpreted in terms of the ability of cADPR to increase the calcium sensitivity of CICR in cardiac muscle. Among the factors considered to account for the marked temperature-dependence of the observed effects was the possibility that actions of cADPR might involve not only the ryanodine-receptor release channel, but also additional proteins, such as putative cADPR binding proteins [9], calmodulin [10,11], calmodulin dependent protein kinase [12] and FK-binding proteins [13]. These proteins and cADPR might then interact with kinetics which are both complex and temperature sensitive.

The aim of the present experiments was to test further these possibilities using caged cADPR. It appears that photoreleased cADPR can increase whole-cell calcium transients, but that the effects are not immediate, taking approx. 15 s to develop under the conditions of our experiments. Since whole-cell calcium transients are thought to represent synchronous activation of many calcium sparks [14], we also wished to investigate whether the actions of cADPR to increase calcium transients might arise from effects of cADPR on the characteristics of individual sparks or on their probability of occurrence. Preliminary observations have been presented previously [15].

MATERIALS AND METHODS

Cell isolation and superfusion

Guinea-pigs or rats were killed by cervical dislocation following stunning, and the hearts were excised. Myocytes were isolated from ventricular muscle after perfusion of the heart with collagenase, using methods which have been described in detail [16,17]. Guinea-pig myocytes were superfused with a solution containing (mM): NaCl, 118.5; NaHCO₃, 14.5; KCl, 4.2; KH₂PO₄, 1.18; MgSO₄·7H₂O, 1.18; CaCl₂, 2.5; glucose, 11.1, pH 7.4 (oxygenated at 95% O₂/5% CO₂). A similar solution was used for measurement of calcium sparks in rat myocytes, except that the calcium concentration was reduced to 1.0 mM to avoid calcium waves which tended to occur spontaneously in the absence of electrical stimulation at higher calcium concentrations in this species. All experiments (including those combining electrophysiology and confocal microscopy) were carried out at 36 °C.

Abbreviations used: cADPR, cADP-ribose; CICR, calcium-induced calcium release.

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Electrophysiology

Whole-cell patch techniques were used for electrical recording (Axoclamp 2B). When whole-cell calcium transients were to be recorded, action potentials were stimulated by application of current pulses of 2 ms duration [8,18,19]. A pulse interval of 2 s was chosen to allow synchronized linescan images to be obtained on the confocal microscope as described below. The pipette solution contained (mmol/l): KCl, 140; NaCl, 5; MgCl₂, 2; K₂ATP, 1; Hepes, 5 (pH 7.2). Fluo-3 (100 μ M) and caged cADPR (1 mM) were added to the pipette solution.

Confocal microscopy

Whole-cell calcium transients and calcium sparks were recorded in linescan mode, in which the excitation laser repeatedly scanned along a line positioned through the long axis of the ventricular cell. A Leica TCS NT system coupled to a Leica DMIRB inverted microscope with a 63 $\times$ water-immersion objective was used for these experiments. In Figures 1(a) and 1(b), and 3(a) and 3(b), changes in fluorescence with distance along the cell are represented left to right, and time (with repeated linescans) is represented top to bottom. Under the conditions of the experiments the time between the start points of successive lines was 2.6 ms. Excitation of fluo-3 was at 488 nm (argon laser). Fluorescence was detected at wavelengths longer than 515 nm (using a long-pass cut-off filter at this wavelength). A second laser, which provided light at wavelengths of 351 nm and 364 nm, was used for photolysis of caged cADPR. This 'uncaging' light was applied for periods between 500 and 800 ms, and was timed to occur with a delay of 10 ms between the end of the uncaging light and application of the current stimulus (2 ms duration) to initiate an action potential in a ventricular cell. A foot switch allowed control of the output of the laser so that only one period of uncaging light was applied, followed by a series of action potentials for which fluorescence transients were recorded. The stimulation rate for these experiments was 0.5 Hz. The interval between stimuli was determined by the scanning software for the confocal microscope, which in turn triggered the external stimulator controlling current stimuli for initiating action potentials. This synchronizing arrangement allowed the vertical position (time) for initiation of action potentials to be placed consistently in successive linescan images. In experiments where calcium sparks were recorded in rat cells, the myocytes were unstimulated, and a similar procedure to that described above was used to control the opening of the shutter to provide a single 500 ms application of uncaging light at 351 nm and 364 nm.

Statistics

Student's *t*-tests (paired or two-sample, as appropriate) were used to evaluate statistical significance (effects taken to be significant when $P < 0.05$).

RESULTS AND DISCUSSION

Figure 1 shows linescan images of a guinea-pig cell before (a) and after (b) photorelease of cADPR. Each image is constructed from a sequence of fluorescence-intensity plots across the cell, so that distance along the cell is shown from left to right, and time (with 2.6 ms between lines) is shown from top to bottom. After a delay of 510 ms from the start of image acquisition, an action potential was stimulated by application of a 2 ms current pulse through the patch electrode, and the resulting action potential triggered the increase in fluo-3 fluorescence, which is evident as a sudden increase in light intensity which then subsides (reflecting the calcium transient). The second image (b) shows a similar

sequence 16 s after photorelease of cADPR by a 500 ms exposure to UV light, which was timed to occur before one action potential in a series of similar records (10 ms interval between the end of the UV exposure and the stimulus for the first action potential in the series). It can be seen that photorelease of cADPR led to an increase in peak fluo-3 fluorescence. This is illustrated more quantitatively in Figure 1(c), which shows the time course of calcium transients calculated from the fluo-3 fluorescence averaged across the cell in a series of similar images. For each trace the stimulus to fire an action potential was applied 510 ms after the start of the frame (196 lines at 2.6 ms per line); each calcium transient shows an abrupt rise, reflecting CICR from the sarcoplasmic reticulum triggered by calcium entering across the sarcolemma during the action potential. The calcium transient with the smallest peak fluorescence corresponds to the first image in the series (before application of UV light), and successive transients show a gradual increase in peak amplitude (2 s between traces) until a maximal effect is seen approx. 14 s after the UV light.

Similar effects were observed in a series of experiments of this kind on guinea-pig ventricular myocytes. The mean increase in fluo-3 fluorescence caused by photorelease of cADPR under these conditions was $29 \pm 7\%$ ($n = 8$ cells; $P < 0.05$). The time course of the increase in peak calcium transient measured in this way in a series of experiments is shown in Figure 2; each point represents the mean peak calcium transient measured from fluorescence traces similar to those shown in Figure 1(c). It can be seen that the slow development of the increase in peak fluo-3 fluorescence over a period of approx. 15 s was a consistent finding. No detectable changes in the peak calcium transient were seen when cells which had not been loaded with caged cADPR were exposed to UV light (Figure 2).

If the effects of photoreleased cADPR on the calcium transient were on the postulated agonist-binding site, leading to a change in calcium sensitivity of the ryanodine receptor, these effects would be expected to be suppressed by 8-amino-cADPR, a competitive antagonist of both cADPR-induced calcium release and binding in sea urchin egg microsomes and in other systems [20], which has been shown to reduce the effects of cADPR on contraction of cardiac myocytes [8,18]. This possibility was tested in another series of experiments in which guinea-pig ventricular myocytes were loaded with 5 μ M 8-amino-cADPR in addition to fluo-3 and caged cADPR. Under these conditions, photorelease of cADPR, using UV exposures similar to those described above, failed to increase the magnitude of calcium transients ($4 \pm 4\%$; $n = 9$ cells; $P > 0.05$). The mean peak calcium transients following photorelease of cADPR under these conditions are shown in Figure 2, and contrast with the increase in calcium transients observed after photorelease of cADPR in the absence of 8-amino-cADPR. Taken together, these observations are therefore consistent with the ability of photoreleased cADPR to increase the amplitude of calcium transients in guinea-pig cardiac ventricular myocytes by an action at a site or sites which can be antagonized by 8-amino-cADPR.

The possible effect of photoreleased cADPR on calcium sparks was investigated in another series of experiments in rat ventricular myocytes. Calcium sparks have been proposed to represent elementary units of calcium release from the sarcoplasmic reticulum [14], so that a whole-cell transient would be made up of many spark-like events occurring simultaneously (see [21,22] for a detailed discussion of hierarchies of calcium signalling events). Rat myocytes were chosen for these experiments since it was found that sparks were rarely seen in resting guinea-pig cells. It was first necessary to test for the effects of photoreleased cADPR on calcium transients in rat ventricular myocytes. Again,

Figure 1 Effects of photoreleased cADPR on calcium transients in isolated guinea-pig ventricular myocytes

Line-scan images of a guinea-pig cell before (a) and after (b) photorelease of cADPR. The cell was stimulated at 0.5 Hz to provoke the calcium transients. The scan lines were stacked in a top-to-bottom order. Scale bars, vertical, 200 ms; horizontal, 5 μ m. In each of these panels, stimuli to provoke action potentials were applied at a time corresponding to the abrupt increase in fluorescence intensity (marked by horizontal arrows). (c) Time course of calcium transients calculated from line-scan images such as those in (a) and (b). Each point was obtained by averaging the fluo-3 fluorescence of each scanned line. The time axis shows the number of lines, with the time between lines corresponding to 2.6 ms. The stimulus to fire the action potential was applied at 510 ms or 196 lines, immediately preceding the abrupt increase in intensity representing the rising phase of the calcium transient. The calcium transient with the smallest peak fluorescence corresponds to the first image before application of UV light, and successive transients show a gradual increase in peak amplitude after the application of UV light.

Figure 2 Time course of effects of photoreleased cADPR on calcium transients

Changes in calcium transients before and after UV light in the absence (UV control, ●), presence of caged cADPR alone (■), or caged cADPR plus 8-amino-cADPR (◆). Each point represents the averaged peak amplitude of fluorescence (measured from traces similar to those shown in Figure 1c) in 8–9 cells.

an increase in calcium transient was observed, and, in this species, the mean increase in peak fluo-3 calcium transient after photorelease of cADPR was $16 \pm 2\%$ ($n = 6$ cells). Calcium sparks in rat ventricular myocytes were then investigated and

Figure 3 shows linescan images similar to those in Figure 1, except that these cells were not stimulated to fire action potentials and therefore did not show whole-cell calcium transients. One calcium spark, shown by the localized increase in fluo-3 fluorescence, is evident in Figure 3(a). After photorelease of cADPR by application of UV light (exposure period 500 ms, similar to that used in the experiments on whole-cell transients) there was an increase in the frequency of sparks, as illustrated in Figure 3(b) in which a series of sparks can be detected. In a series of similar experiments, photorelease of cADPR caused a consistent increase in spark frequency from 1.2 ± 0.4 number per s per 100 μ m to 2.6 ± 0.5 number per s per 100 μ m ($n = 5$ cells; $P < 0.05$). The increase is shown in Figure 3(c).

As was the case with the whole-cell calcium transients reported above, the increase in spark frequency after photorelease of cADPR would be expected to be sensitive to 8-amino-cADPR. In a series of cells loaded with 8-amino-cADPR, in addition to fluo-3 and caged cADPR, photorelease of cADPR by UV exposures similar to those described above failed to increase the frequency of sparks (1.2 ± 0.3 number per s per 100 μ m before photorelease of cADPR and 0.9 ± 0.2 number per s per 100 μ m after photorelease of cADPR, $n = 6$ cells; $P > 0.05$). The lack of effect of photoreleased cADPR on spark frequency in the presence of 8-amino-cADPR is shown in Figure 3(c).

In addition to increasing spark frequency, it is possible that photoreleased cADPR might modify the characteristics of individual sparks. The spark characteristics which were measured were: amplitude (F/F_0), time to reach a peak (rise time, ms), time constant of decay of fluorescence towards the baseline level (time constant of decay, ms) and width of the spark at a level of

Figure 3 Effects of photoreleased cADPR on calcium-spark frequency in isolated rat ventricular myocytes

Line-scan images of a rat cell before (a) and after (b) photorelease of cADPR. The scan lines were stacked in a top-to-bottom order. Scale bars: vertical, 200 ms; horizontal, 5 μm . (c) Averaged changes in calcium-spark frequency induced by photoreleased cADPR in the absence and presence of 8-amino-cADPR. Black bars, before UV light; grey bars, after UV light. * $P < 0.05$ for comparison between mean spark amplitude before and after UV light to photorelease cADPR. Results are given as means $\pm$ S.E.M., $n = 5$ –6 cells.

Figure 4 Effects of photoreleased cADPR on calcium-spark properties

Histograms of calcium spark amplitude (a), time to the peak (b), time constant of decay (c) and full width at half magnitude (FWHM) (d) before (open bars) and after (bars with diagonal shading) photorelease of cADPR.

fluorescence equal to half the peak (FWHM, μm). Distributions of these spark characteristics before and after photorelease of cADPR are shown in Figure 4. No significant changes ($P > 0.05$) were detected in amplitude (1.9 ± 0.1 times baseline fluorescence before and 1.8 ± 0.1 times after cADPR), rise time (17 ± 2 ms before and 16 ± 1 ms after cADPR), time constant of decay (26 ± 2 ms before and 30 ± 1 ms after cADPR) or distance to half amplitude (2.1 ± 0.1 μm before and 2.1 ± 0.1 μm after cADPR).

The main findings reported here are an increase in the whole-cell calcium transient, which takes many seconds to develop after

photorelease of cADPR, and a cADPR-induced increase in spark frequency with little or no change in the characteristics of individual sparks. These experiments therefore provide the first evidence for an effect of cADPR on calcium sparks in any cell type and are the first to demonstrate actions of photoreleased cADPR on calcium transients in any mammalian cell.

In the case of guinea-pig ventricular cells, these observations provide further evidence that cytosolic cADPR can enhance calcium transients at 36 °C. In our earlier work using the calcium indicator fura-2, the increase in peak calcium transient, following cytosolic application of cADPR via a patch pipette, occurred without any obvious effect on the decay of the calcium transient, and there appeared to be an enhancement of the initial rapid rising phase of the transient, leading to an increased amplitude of the peak [19]. The observations reported here using fluo-3, an indicator with lower affinity for calcium, and therefore with a reduced tendency for calcium buffering and slowing of calcium transients [23], were broadly similar.

The slow development of the effects of photoreleased cADPR on whole-cell calcium transients over a period of approx. 15 s may provide important clues concerning its mechanism of action. It is worth noting that the time course for the development of an increase in spark frequency after photorelease of cADPR appeared to be similarly slow, indicating that the same cellular mechanism may lead to the two effects, and that a sequence of action potentials, which could for example be associated with changes in stores loading, is not required. One possible explanation for the slow time course could be the possible complex interactions of cADPR with the proteins controlling CICR. The actions of cADPR might not involve simple direct binding of cADPR to the ryanodine-receptor release channel, but might involve additional proteins such as calmodulin [10,11,24], calmodulin kinase II [12], FK-binding protein [13] or putative cADPR-binding proteins [9]. Complex interactions of this kind provide the opportunity for delays to occur (although protein–protein interactions need not necessarily be slow), and could account for the marked temperature-dependence of cADPR actions, as reported previously [19].

The increase in the frequency of calcium sparks, with little or no change in their amplitude or time course following photorelease of cADPR, shows that, at least at diastolic levels of calcium, the predominant effect of cADPR is to enhance the probability of occurrence of sparks, presumably by increasing the effectiveness of calcium to cause calcium release from the sarcoplasmic reticulum. If this enhancement by cADPR of the probability of spark occurrence were also to occur at systolic levels of calcium, the observed increase in whole-cell calcium transients would be expected to occur. The physiological function of cADPR in cardiac ventricular muscle may therefore be to increase the 'gain' of CICR (increasing the probability of spark occurrence for a given level of cytosolic calcium), so that an increased calcium transient, which can be seen as the synchronous occurrence of many spark-like events triggered by the entry of calcium across the surface membrane, leads to an increased contraction. Recent preliminary evidence shows that the synthesis of cADPR is enhanced by protein kinase A [25], raising the possibility that elevation of cADPR levels may be added to the variety of mechanisms leading to the positive inotropic actions of β -adrenoceptor agonists. It also appears that an excess of cADPR may increase the gain of CICR to such an extent that arrhythmogenic mechanisms are provoked [26].

One point to consider is whether an effect of cADPR on CICR is the only mechanism of action leading to an increase in calcium transients and contractions. For example, it has been suggested that agents which enhance calcium release from the sarcoplasmic reticulum may not cause maintained effects on calcium transients and contractions because there is a compensating reduction in the loading of the sarcoplasmic reticulum with calcium [27]. Evidence has been presented that this appears to be the case, at least for low doses of caffeine in rat cells at room temperature [27]. The extent of depletion of the sarcoplasmic reticulum is expected to depend on the balance between diastolic and systolic effects of the drug and on the influence of competing mechanisms of calcium uptake and extrusion. In guinea-pig cells, a major component of calcium re-uptake into the sarcoplasmic reticulum stores occurs during the positive plateau of the action potential, when extrusion of calcium through sodium exchange is greatly reduced, therefore favouring re-uptake of calcium into the sarcoplasmic reticulum. Under these conditions, if a greater fraction of calcium were released from the sarcoplasmic reticulum as a consequence of the sensitizing action of cADPR, the majority of released calcium would be expected to be taken up again so that depletion is minimal. Stores depletion might nevertheless occur if cADPR promotes loss of calcium during diastole. Some effect of this kind presumably does occur, since spark frequency was shown to be enhanced, at least in rat cells, though the lack of substantial reduction of spark amplitude appeared to indicate that such an effect was not large. However, if cADPR were to sensitize CICR to calcium this need not arise from a simple shift in the log [concentration]–response curve relating probability of release to log [calcium concentration]. A greater sensitizing effect of cADPR at systolic than at diastolic levels of calcium might allow a maintained effect of this agent on whole-cell calcium transients under the conditions of our experiments, as observed in guinea-pig ventricular cells. In this context, we have found that 200 μ M caffeine [28] had a substantially larger effect on spark frequency (an approx. 6-fold increase rather than a doubling) than that reported above for photoreleased cADPR, and caused a small reduction in spark amplitude, perhaps because of store depletion associated with the greater frequency of sparks. The effects of this dose of caffeine on contractions

accompanying action potentials were not greater than those of photoreleased cADPR in guinea-pig cells (Y. Cui, A. Galione and D. A. Terrar, unpublished work). These observations might be taken to indicate a difference between caffeine and cADPR in the balance between diastolic and systolic effects, with cADPR showing relatively greater effects during systole as suggested above, though more experiments will be necessary to investigate this possibility further. The molecular sites of action of cADPR and caffeine in sensitizing ryanodine receptors are expected to differ, since 8-amino-cADPR blocks cADPR, but not caffeine-induced calcium release, in sea urchin egg homogenates [20]; caffeine does, however, potentiate cADPR-induced calcium release in this system [29].

In summary, these observations support a role for cADPR in regulating ryanodine-receptor function in cardiac myocytes. The effects were also evident at the level of calcium sparks. However, the pronounced latency of cADPR-induced potentiation of CICR through ryanodine-receptor openings might be taken to indicate that the influence of cADPR on CICR is more complex than originally envisaged, perhaps involving additional molecular interactions.

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