An Antagonist of cADP-ribose Inhibits Arrhythmogenic Oscillations of Intracellular Ca²⁺ In Heart Cells

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Oscillations of Ca²⁺ in heart cells are a major underlying cause of important cardiac arrhythmias, and it is known that Ca²⁺-induced release of Ca²⁺ from intracellular stores (the sarcoplasmic reticulum) is fundamental to the generation of such oscillations. There is now evidence that cADP-ribose may be an endogenous regulator of the Ca²⁺ release channel of the sarcoplasmic reticulum (the ryanodine receptor), raising the possibility that cADP-ribose may influence arrhythmogenic mechanisms in the heart. 8-Amino-cADP-ribose, an antagonist of cADP-ribose, suppressed oscillatory activity associated with overloading of intracellular Ca²⁺ stores in cardiac myocytes exposed to high doses of the β-adrenoceptor agonist isoproterenol or the Na⁺/K⁺-ATPase inhibitor ouabain. The oscillations suppressed by 8-amino-cADP-ribose included intracellular Ca²⁺ waves, spontaneous action potentials, after-depolarizations, and transient inward currents. Another antagonist of cADP-ribose, 8-bromo-cADP-ribose, was also effective in suppressing isoproterenol-induced oscillatory activity. Furthermore, in the presence of ouabain under conditions in which there was no arrhythmogenicity, exogenous cADP-ribose was found to be capable of triggering spontaneous contractile and electrical activity. Because enzymatic machinery for regulating the cytosolic cADP-ribose concentration is present within the cell, we propose that 8-amino-cADP-ribose and 8-bromo-cADP-ribose suppress cytosolic Ca²⁺ oscillations by antagonism of endogenous cADP-ribose, which sensitizes the Ca²⁺ release channels of the sarcoplasmic reticulum to Ca²⁺.

The release of Ca²⁺ from the sarcoplasmic reticulum (SR),¹ mediated by Ca²⁺ release channels known as ryanodine receptors (RyRs), is believed to play an important role not only during normal cardiac muscle contraction but also during abnormal conditions associated with Ca²⁺ overload and oscillations of cell Ca²⁺ and membrane potential (1). These oscillations of intracellular Ca²⁺ are thought to arise from cyclical release and reuptake of Ca²⁺ by the SR stores and have been suggested to underlie a variety of disturbances of the rhythm of the heart (cardiac arrhythmias).

Ryadanine, which is known to interfere with the function of the SR by an action at RyRs, suppresses oscillations of Ca²⁺ in cardiac myocytes, demonstrating the important role of the RyR and Ca²⁺-induced Ca²⁺ release (CICR) in sustaining spontaneous activity (2). In recent years, cADP-ribose (cADPR) has emerged as a possible endogenous regulator of RyR function by enhancing the sensitivity of CICR to Ca²⁺ (3–6). Enzymes for the synthesis and breakdown of cADPR are present in cardiac muscle (7–10), and endogenous levels have been estimated to be approximately 200 nM (11). Studies in intact heart cells have yielded results consistent with a role for endogenous cADPR in the regulation of excitation-contraction coupling. In guinea pig cardiac myocytes stimulated to fire action potentials, Ca²⁺ transients and contractions are enhanced by intracellular applications of cADPR (12) and reduced by antagonists of cADPR, 8-amino-cADPR and 8-bromo-cADPR (12–17). These observations are consistent with endogenous cADPR, acting to enhance the Ca²⁺ sensitivity of CICR, an action that may be antagonized by 8-amino-cADPR and 8-bromo-cADPR.

In this study we have investigated (in guinea pig ventricular cells) the possible influence of 8-amino-cADPR and 8-bromo-cADPR on spontaneous release of Ca²⁺ from the SR under conditions of Ca²⁺ overload. The two maneuvers chosen to induce Ca²⁺ oscillations involved the use of isoproterenol and ouabain to generate Ca²⁺ overload. In addition, we have examined whether exogenous application of cADPR may itself be arrhythmogenic under certain conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation**—Myocytes were isolated enzymatically from guinea pig ventricle as described previously (18, 19) and superfused at 34–36 °C with a solution containing 118.5 mM NaCl, 14.5 mM NaHCO₃, 4.2 mM KCl, 1.18 mM KH₂PO₄, 1.18 mM MgSO₄·7H₂O, 2.5 mM CaCl₂, 11.1 mM glucose (oxygenated at 95% O₂, 5% CO₂).

**Electrophysiology**—In experiments investigating the actions of 8-amino-cADPR, cells were impaled with double-barreled “theta glass” sharp microelectrodes, allowing one barrel (containing 1 M potassium methyl sulfate + 10 mM KCl) to be used for electrical recording and injection of the other for cytosolic application of drugs (after obtaining a series of measurements in the absence of drug). Drugs were applied to the cell during the course of an experiment by injecting a small quantity of the appropriate solution into the previously empty barrel of the electrode; this traveled to the tip of the electrode by capillary action and entered the cell by diffusion. In experiments investigating the actions of cADPR, electrical recordings were made from cells using conventional whole-cell (ruptured patch) patch clamp techniques. When drugs were applied to the cytosol, these were included in the pipette solution. Control records were taken in the first min, when it was believed that dialysis from the pipette to the cytosol was minimal, and compared with records 5 to 10 min after rupture of the membrane, when access of the compound to the cytosol was thought to be well established. An Axoclamp 2A recording system was used for switched voltage clamp.

**Generation of Oscillatory Activity with Isoproterenol**—Spontaneous contractile and electrical activity was induced by exposure of cells to isoproterenol (20–100 nM, applied continuously). In preliminary experiments, it was found that continuous superfusion with 20 nM isoprot...
FIG. 1. Effects of 8-amino-cADPR on spontaneous action potentials provoked by isoproterenol. A, intracellular records of membrane potential recorded from a ventricular myocyte isolated from guinea pig heart. Action potentials were evoked by electric current stimuli (2-ms duration) applied at the arrows. B, records in the same cell as for panel A showing spontaneous action potentials provoked by application of (−)-isoproterenol (20 nM). Note the lack of correlation, with arrows marking current stimuli. C, suppression of the spontaneous activity (with action potentials occurring only at the time of the current stimuli applied at the arrows) by 8-amino-cADPR (20 μM) dissolved in HEPES buffer (20 mM) and applied to the cytosol through one barrel of a microelectrode made using theta glass tubing. D, action potentials recorded in another cell under the same conditions as for panel A. E, spontaneous activity recorded in the same cell shown in panel D following application of isoproterenol (i.e., same conditions as for panel B). F, activity in the same cell as for panels D and E recorded 5 min after cytosolic application of HEPES buffer without 8-amino-cADPR. Note that spontaneous activity persisted under these conditions.

8-amino-cADPR. Note that spontaneous activity persisted under these conditions. 

Ouabain-induced spontaneous activity was monitored using a voltage clamp protocol of trains of 5 200-ms step depolarizations (from −70 mV to +40 mV) at a frequency of 2.5 Hz, with a 7-s interval between trains. With these protocols, no spontaneous activity was observed in the absence of ouabain.

**Generation of Oscillatory Activity with Ouabain—**Spontaneous contractile and electrical activity was induced by exposure of cells to ouabain (1 μM, applied for 6–10 min and then washed away). Continuous superfusion with 1 μM ouabain throughout the course of an experiment did not produce stable oscillatory behavior but caused the generation of oscillations, which increased in severity with time, resulting in the gradual deterioration of the cell; however, ouabain-provoked oscillations, once induced, were found to be stable for a period exceeding 8 min after washout of ouabain. For this reason, in experiments investigating the effects of drugs on ouabain-induced oscillations, superfusion with ouabain was discontinued before cytosolic injection of drugs (injection of drugs was carried out within 1 min of washout of ouabain). Ouabain-induced spontaneous activity (in cells not loaded with fura-2) was monitored using a voltage clamp protocol of trains of 5 200-ms step depolarizations (from −70 mV to +40 mV) at a frequency of 1 Hz). In a further series of experiments, oscillations of intracellular Ca2+ induced by ouabain were monitored directly from the fluorescence of fura-2. Cells loaded with fura-2 were found to be less susceptible to the arrhythmogenic actions of ouabain, possibly because of the Ca2+-buffering action of the Ca2+ indicator, and hence different conditions were employed from those used to study myocytes not loaded with the Ca2+ dye. Cells were superfused with 120 mM isoproterenol, and the voltage clamp protocol consisted of trains of 20 200-ms step depolarizations from −70 mV to +40 mV at a frequency of 3.3 Hz, with a 5-s interval between trains. With these protocols, no spontaneous activity was recorded in the absence of isoproterenol.

**Intracellular Free Ca2+ Measurements Using Fura-2—**Fura-2 fluorescence was monitored from cells preincubated with the acetoxymethyl ester of fura-2 (5 μM) for 15–20 min; after loading, a period of at least 30 min was allowed before experimentation for deesterification of the intracellularly accumulated fura-2-AM. Excitation light (wavelength 340 ± 5 nm) was delivered by means of a fiberoptic (diameter 125 μm) with enhanced ultraviolet transmission positioned very close to the cell under study; emitted fluorescence light (500 ± 40 nm) was collected through the microscope objective and quantified by means of a photomultiplier tube.

**Imaging of Ca2+ Waves Using Fluo-3—**Fluo-3 fluorescence was monitored from cells preincubated with the acetoxymethyl ester of fluo-3 (5 μM) for 15–20 min; after loading, a period of at least 30 min was allowed before experimentation for deesterification of the intracellularly accumulated fluo-3-AM. Excitation light (wavelength 485 ± 11 nm) from a xenon arc lamp (75 W) was delivered by means of a fiberoptic (diameter 125 μm) with enhanced ultraviolet transmission positioned very close to the cell under study; emitted fluorescence light (530 ± 15 nm) was collected through the microscope objective and captured with a Photonic Science Isis III-intensified CCD camera.

**Confocal Microscopy—**Myocytes imaged under the confocal microscope were loaded with fluo-3 as described above. A Leica TCS NT confocal scanning head was coupled to a DMRB microscope with a 63× water immersion objective lens. Illumination was provided by a 488-nm Ar laser, and a 515-nm long pass filter was used in the collection of emitted fluorescence. Line scan imaging was used to maximize temporal resolution; a single line along the long axis of the heart cell was...
repeatedly scanned at an acquisition rate of 385 Hz, and images were constructed by displaying successive lines (corresponding to 66 μm length) adjacent to each other. Statistics—Values are expressed as mean ± S.E.

RESULTS

Although low doses of isoproterenol (5 nM) cause an approximate doubling of Ca²⁺ currents and contractions in guinea pig ventricular cells with no initiation of spontaneous activity, high doses (20 nM and higher) provoke spontaneous electrical activity. Fig. 1 illustrates spontaneous electrical activity recorded from a representative cell exposed to 20 nM isoproterenol and stimulated to fire action potentials at a frequency of 1 Hz. In the absence of any drugs (Fig. 1A), only stimulated action potentials were recorded, and no spontaneous electrical activity was observed. However, subsequent exposure to isoproterenol resulted initially (1–2 min) in the appearance of small transient depolarizations between stimulated action potentials (after-depolarizations), which increased in magnitude until sufficiently large to elicit spontaneous action potentials (each associated with a spontaneous contraction). Examples of spontaneous action potentials recorded in this cell after a 3-min superfusion with isoproterenol are shown in Fig. 1B. Fig. 1C shows the effect of cytosolic infusion of 8-amino-cADPR (pipette concentration of 20 μM, dissolved in 20 mM HEPES buffer) in the continued presence of isoproterenol. Within 3 min of 8-amino-cADPR injection, spontaneous electrical activity was completely abolished in this cell, whereas the generation of stimulated action potentials was not affected. Similar experiments were carried out in a total of 8 cells. In 6 of these, isoproterenol-induced spontaneous electrical activity was completely (4 cells) or partially (2 cells) inhibited by 8-amino-cADPR within 5 min. No apparent influence of 8-amino-cADPR was observed in the remaining 2 cells. Overall, the number of spontaneous events recorded over a 5-s period was significantly reduced from 6.4 ± 0.7 to 2.8 ± 1.2 (p < 0.05; n = 8) within 5 min of 8-amino-cADPR infusion.

In the cells in which 8-amino-cADPR suppressed spontaneous activity, these actions appeared to develop progressively with time; spontaneous action potentials were superceded by after-depolarizations, and after-depolarizations appeared to gradually decrease in magnitude and occur with progressively increasing delay after the preceding stimulated action potential. Such a progressive suppression of oscillatory activity may reflect a gradual increase of 8-amino-cADPR concentration within the cell.

As a control for the above series of experiments, the effects of injection of HEPES buffer (20 mM) in the absence of 8-amino-cADPR were investigated. In 6 of 9 cells studied, little or no effect of HEPES was observed on isoproterenol-induced spontaneous electrical activity (data from a representative cell are shown in Fig. 1, D, E, and F). In 2 cells, a slight increase in the

Fig. 2. Effects of 8-amino-cADPR on transient inward currents provoked by isoproterenol. A, records of spontaneous activity (initiated by isoproterenol) recorded under voltage clamp conditions (switched voltage clamp). The lower trace in this and later panels represents the membrane potential, clamp steps to +40 mV for 200 ms from a holding potential of −70 mV, frequency 1 Hz. Exposure of the cells to isoproterenol (20 nM) provoked transient inward currents (upper trace) which can be seen as fluctuations in the current level between pulses (arrow). B, these spontaneous transient inward current fluctuations were suppressed by 8-amino-cADPR (records in same cell as for panel A, recorded 5 min after the addition of 20 μM 8-amino-cADPR to the application barrel of the microelectrode). C, spontaneous activity (arrow) in another cell under the same conditions as for panel A. D, failure of cytosolic application of 20 mM HEPES buffer to suppress spontaneous transient inward currents (traces in the same cell as for panel C were recorded 5 min after the addition of HEPES to the application barrel of the microelectrode).

a S. Rakovic, Y. Cui, S. Iino, A. Galione, G. A. Ashamu, B. V. L. Potter, and D. A. Terrar, unpublished data.
frequency of occurrence of spontaneous activity was observed, whereas in 1 cell, a small decrease was noted. Overall, HEPES was without significant effect; the number of spontaneous events recorded before and 5 min after injection of HEPES was 6.4 ± 0.6 and 6.9 ± 0.7, respectively (p > 0.05; n = 9).

In a further series of experiments, the effects of 8-amino-cADPR and HEPES on isoproterenol-induced oscillations were investigated under voltage clamp conditions to avoid any effects that might arise from changes in action potential duration. Cells were stimulated at a frequency of 1 Hz with 200-ms step depolarizations from a holding potential of −70 mV to +40 mV. In all cells studied, superfusion with 20–100 nM isoproterenol for 3 min resulted in the appearance of transient inward currents between depolarizing pulses; a representative example is presented in Fig. 2. These transient inward currents are likely to be secondary to spontaneous Ca\(^{2+}\) release from an overloaded SR and are thought to be carried predominantly by sarcolemmal Na\(^{+}/Ca^{2+}\) exchange operating in the Ca\(^{2+}\) extrusion mode (20, 21). In this cell, subsequent infusion of 20 μM 8-amino-cADPR (in the continued presence of isoproterenol) was associated with complete suppression of transient inward currents within 5 min (Fig. 2B). In a total of 9 cells investi-
Ca²⁺ susceptible to exhibit spontaneous oscillations in cytosolic fura-2, using a photomultiplier tube to collect emitted fluorescence. These oscillations of cytosolic Ca²⁺ initiated near the lower end of the image and subsequently propagated along the scanned line (and hence cell).

In the absence of isoproterenol, the fluorescence signal was stable between trains of voltage clamp pulses; in the presence of isoproterenol. Data from a typical cell are presented in Fig. 3, A, showing intracellular records of membrane potential recorded from a single ventricular myocyte superfused for 3 min with isoproterenol (50 nM). A series of 10 action potentials was evoked by electric current stimuli (2 ms duration) applied at the blue lines, and this was subsequently followed by a single after-depolarization (arrow). The lower panel displays line scan images obtained at the corresponding times. Each image is composed of 512 scanned lines (66 μm in length, scan rate 385 Hz) presented in a vertical orientation. Hence, each image has a vertical dimension of 66 μm and a horizontal dimension of 1331 ms. Each stimulated action potential is accompanied by a rapid rise in intracellular Ca²⁺ (red), which occurred uniformly along the scanned line (images 1–6). Image 8 illustrates a Ca²⁺ wave that was associated with the after-depolarization; the wave was initiated near the lower end of the image and subsequently propagated along the scanned line (and hence cell). B, data from the same cell, recorded 3 min after infusion of 8-amino-cADPR (20 μM) in the continued presence of isoproterenol. No spontaneous activity was present, as illustrated by the lack of after-depolarizations and Ca²⁺ waves.

gated, 5 showed complete suppression of transient inward currents within 5 min of 8-amino-cADPR infusion; in 2 cells, the magnitudes of transient inward currents were reduced, whereas in a further 2 cells, there was little or no change. Overall, 8-amino-cADPR (5 min) suppressed the frequency of occurrence of transient inward currents from 0.84 ± 0.08/step depolarization to 0.33 ± 0.07 (p < 0.05; n = 9) and reduced the magnitude of the first transient inward current to 42 ± 7%, that of the preinjection value (p < 0.05; n = 9).

In contrast, infusion of HEPES did not reduce the frequency of occurrence and magnitudes of isoproterenol-induced transient inward currents in 5 cells studied. Results from a representative cell are presented in Fig. 2, C and D.

To monitor more directly isoproterenol-induced Ca²⁺ oscillations, experiments similar to those described above were repeated in cells loaded with the fluorescent Ca²⁺ indicator fura-2, using a photomultiplier tube to collect emitted fluorescence. It was found that cells loaded with fura-2 were not as susceptible to exhibit spontaneous oscillations in cytosolic Ca²⁺, possibly because of the buffering action of the Ca²⁺ indicator, and hence more vigorous conditions were required to generate oscillatory changes in Ca²⁺ (see the Fig. 3 legend). In 5 of 7 cells studied, complete cessation of isoproterenol-provoked Ca²⁺ oscillations occurred within 6 min of 8-amino-cADPR injection (20 μM) despite continuous superfusion with isoproterenol. Data from a typical cell are presented in Fig. 3. In the absence of isoproterenol, the fluorescence signal was stable between trains of voltage clamp pulses; in the presence of isoproterenol, transient deflections in the fluorescence signal were recorded between trains of voltage clamp pulses, representing spontaneous elevations of intracellular Ca²⁺ (Fig. 3A). These oscillations of cytosolic Ca²⁺ were abolished within 6 min of 8-amino-cADPR infusion (Fig. 3B). In the remaining 2 cells, 8-amino-cADPR infusion was associated with prolongation of the time to onset of the spontaneous event, with little change in the magnitude. In these 7 cells, injection of 8-amino-cADPR was associated with significant (p < 0.05) reductions both in the number of spontaneous oscillations per train of voltage clamp pulses (from 1.14 ± 0.14 to 0.29 ± 0.18) and in the peak magnitude of the first oscillation following a train of pulses, as determined from the fura-2 fluorescence signal (to 27 ± 18% that before 8-amino-cADPR injection). In six control experiments, HEPES was not effective in suppressing isoproterenol-induced oscillations under these conditions (data from a representative cell are shown in Fig. 3, C and D).

In another series of experiments, isoproterenol-provoked spontaneous Ca²⁺ waves propagating across the cell were recorded using an image-intensified CCD camera. An example of a spontaneous Ca²⁺ wave, initiated after superfusion with isoprenaline, is shown in Fig. 4A; two waves begin near the center of the cell and propagate toward the edges in opposite directions. In this cell, cytosolic application of 8-amino-cADPR was associated with complete abolition of Ca²⁺ waves within 5 min (Fig. 4B). In 12 of 17 cells studied in this way, Ca²⁺ waves were abolished in a marked or markedly suppressed by 8-amino-cADPR (20 μM) within 8 min.

The spatial characteristics of spontaneous activity and its suppression by 8-amino-cADPR were studied in more detail using a laser-scanning confocal microscope operating in line-scan mode (cells loaded with the Ca²⁺ indicator fluo-3). In this configuration, the temporal resolution was maximized by repeatedly scanning at a rate of 2.6 ms/s along a single line (66 μm in length) across the long axis of the heart cell. The lower panel of Fig. 5A illustrates the images obtained by this method. Each image (numbered 1–12) is composed of 512 sequential line scans with each line displayed in a vertical orien-

**Fig. 5. Effects of 8-amino-cADPR on isoproterenol-provoked Ca²⁺ waves, imaged using a confocal microscope.** A, the upper panel shows intracellular records of membrane potential recorded from a single ventricular myocyte superfused for 3 min with isoproterenol (50 nM). A series of 10 action potentials was evoked by electric current stimuli (2 ms duration) applied at the blue lines, and this was subsequently followed by a single after-depolarization (arrow). The lower panel displays line scan images obtained at the corresponding times. Each image is composed of 512 scanned lines (66 μm in length, scan rate 385 Hz) presented in a vertical orientation. Hence, each image has a vertical dimension of 66 μm and a horizontal dimension of 1331 ms. Each stimulated action potential is accompanied by a rapid rise in intracellular Ca²⁺ (red), which occurred uniformly along the scanned line (images 1–6). Image 8 illustrates a Ca²⁺ wave that was associated with the after-depolarization; the wave was initiated near the lower end of the image and subsequently propagated along the scanned line (and hence cell). B, data from the same cell, recorded 3 min after infusion of 8-amino-cADPR (20 μM) in the continued presence of isoproterenol. No spontaneous activity was present, as illustrated by the lack of after-depolarizations and Ca²⁺ waves.
tation. Thus, the horizontal direction in the image represents increasing time (2.6 ms/line, 1331 ms for each image), and the vertical direction represents the distance along the line scan (66 μm for each image).

In this series of experiments, myocytes were repeatedly stimulated to fire trains of 10 action potentials (at 1 Hz) with a 10-s interval between trains and were provoked to exhibit spontaneous Ca\(^{2+}\) waves by superfusion with 50 nM isoproterenol. The upper panel of Fig. 5A shows data from a representative cell and illustrates 10 stimulated action potentials (stimuli applied at blue line) followed by an after-depolarization (arrow) that developed following superfusion with isoproterenol. The lower panel presents the line scan images obtained over this period; dark colors represent low cytosolic Ca\(^{2+}\), whereas red represents high Ca\(^{2+}\). Images 1–6 illustrate elevations in intracellular Ca\(^{2+}\) accompanying each action potential, and it can be seen that the rise in Ca\(^{2+}\) is relatively uniform along the line scan, representing a synchronous global increase in Ca\(^{2+}\). In contrast, image 8 shows a spontaneous Ca\(^{2+}\) wave associated with the after-depolarization; it can be seen that the rise in Ca\(^{2+}\) is not uniform along the line but commences at a point near the lower end of the image before propagating along the length of the cell as time proceeds. Fig. 5B shows data from the same cell 5 min after injection of 20 μM 8-amino-cADPR. 8-Amino-cADPR completely suppressed the generation of Ca\(^{2+}\) waves and after-depolarizations in this cell without abolishing stimulated action potentials or the calcium transients that accompany them. There was also an apparent reduction in the magnitude of the calcium elevations accompanying each action potential; this might be expected, as 8-amino-cADPR has previously been shown to reduce the magnitude of the calcium transient in guinea pig ventricular myocytes (13). However, a contribution of dye loss to the reduction in fluorescence intensity is likely also to contribute.

Data from another cell are shown in Fig. 6. In this cell, isoproterenol-provoked oscillations were more severe, consisting of spontaneous action potentials (Fig. 6A, upper panel). The line scan images (lower panel) reveal that each spontaneous action potential was preceded by a calcium wave that commenced at a point near the center of the scanned line before propagating bidirectionally toward the edges of the cell. Subsequently a global increase in Ca\(^{2+}\) occurred along the line as the spontaneous action potential was initiated. Image 3 of Fig. 6A has been enlarged for clarity: the white arrow indicates a Ca\(^{2+}\) wave preceding a spontaneous action potential. It is interesting to note that the global rise in Ca\(^{2+}\) accompanying the spontaneous action potential was not uniform along the scanned line; instead, the region of the cell to which the wave had previously propagated showed a smaller increase in Ca\(^{2+}\) than adjacent areas. This may be because of selective depletion of intracellular Ca\(^{2+}\) stores in this region of the cell or inactivation of the RyRs involved in the propagation of the wave. After injection of 8-amino-cADPR, the generation of spontaneous action potentials was completely suppressed, as shown in Fig. 6B, although some oscillatory activity persisted in the form of a single after-depolarization (arrow) accompanied by a Ca\(^{2+}\) wave (image 3, enlarged for clarity). In total, 8 cells were imaged in this way; in 6 of these, 20 μM 8-amino-cADPR completely or partially suppressed isoproterenol-induced calcium waves within 8 min of injection.

The actions of 8-amino-cADPR were also investigated using another method to provoke Ca\(^{2+}\) oscillations. In this series of experiments, spontaneous activity was initiated by exposure to the cardiac glycoside ouabain rather than isoproterenol. Ouabain has been employed in a number of studies as an experimental tool for the generation of spontaneous Ca\(^{2+}\) oscillations.
in heart cells (22, 23). Its major mechanism of action is believed to involve binding to the \( K_1 \) binding site of the sarcolemmal \( Na^+/K^+ \)-ATPase and inhibition of its activity, resulting in a secondary rise in the level of intracellular \( Na^+ \) (24–27). This in turn is thought to inhibit the extrusion of \( Ca^{2+} \) via sarcolemmal \( Na^+/Ca^{2+} \) exchange, leading to an elevation of cytosolic \( Ca^{2+} \) and a secondary increase in the quantity of \( Ca^{2+} \) stored in the SR. The spontaneous oscillations of intracellular \( Ca^{2+} \) associated with ouabain toxicity are believed to be because of overloading of the SR with \( Ca^{2+} \); indeed, elevated levels of “luminal” \( Ca^{2+} \) have been reported to enhance the open probability of RyRs studied in planar lipid bilayers (28). Additional mechanisms may also be involved; certain glycosides have been reported to increase \( Ca^{2+} \) entry via sarcolemmal \( Ca^{2+} \) channels (29) and increase the open probability of single RyRs incorporated into artificial lipid bilayers (30). Furthermore, it has recently been reported that ouabain may alter the ion selectivity of sarcolemmal \( Na^+ \) channels to allow \( Ca^{2+} \) entry (“slip mode conductance”), which might contribute to increased cellular \( Ca^{2+} \) loading (31).

Cytosolic injection of 8-amino-cADPR, but not HEPES, was associated with a suppression of ouabain-induced transient inward currents; representative individual experiments are shown in Fig. 7. In 7 cells studied, intracellular application of 8-amino-cADPR (20 \( \mu M \)) resulted in reductions both in the number of transient inward currents per train of voltage clamp pulses (from 1.85 ± 0.48 to 1.00 ± 0.10; \( p < 0.05 \)) and in the magnitude of the first transient inward current following a series of pulses (to 50 ± 6% that before injection; \( p < 0.05 \)). In contrast, injection of HEPES (20 \( \mu M \)) was without significant effect on either of these variables.

8-Bromo-cADPR is another cADPR analogue that has been shown to act as an antagonist of cADPR, albeit with less potency than 8-amino-cADPR (12, 14). We therefore investigated the actions of this compound in experiments similar to those presented in Fig. 1; cells were stimulated to fire action potentials at 1 Hz, and spontaneous activity was induced by 50 nM isoprenaline. In 6 of 8 cells studied, injection of 100 \( \mu M \) 8-bromo-cADPR was associated with suppression or abolition of spontaneous activity (after-depolarizations and spontaneous action potentials), supporting the hypothesis that this is being achieved through an antagonism of the actions of endogenous cADPR.

Because 8-amino-cADPR and 8-bromo-cADPR, antagonists of cADPR, were found in the above experiments to be effective in suppressing \( Ca^{2+} \) oscillations under conditions of \( Ca^{2+} \) overload, it was of interest to determine whether, under certain conditions, exogenous cADPR might trigger oscillatory behavior in previously quiescent cells. In support of this possibility were previous observations that application of 10 \( \mu M \) cADPR via a patch pipette was found to be associated with the development of spontaneous activity in a minority of cells (4 of 15).

To investigate further whether exogenous cADPR might provoke oscillations, ventricular myocytes were superfused (for 6–7 min) with 1 \( \mu M \) ouabain and stimulated to fire action potentials at 1 Hz, conditions that alone do not lead to arrhythmogenesis but which might be expected to increase the tend-
In view of the evidence in sea urchin egg preparations and mammalian cells that 8-amino-cADPR and 8-bromo-cADPR are competitive antagonists of cADPR-induced Ca\textsuperscript{2+} mobilization (14, 17, 32), we propose that suppression of oscillatory activity by these compounds may be because of antagonism of the actions of endogenous cADPR. ADP-ribosyl cyclase activity has been identified in rat cardiac myocytes (7), and intracellular levels of cADPR have been estimated to be of the order of 200 nm (11). Furthermore, cADPR has been reported to enhance CICR in a number of preparations, including guinea pig heart cells (12), sea urchin eggs (33, 34), and neuronal cells (5, 35–37). This would support the hypothesis that suppression of Ca\textsuperscript{2+} oscillations (provoked by isoproterenol and ouabain) by 8-amino-cADPR and 8-bromo-cADPR is because of antagonism of the actions of endogenous cADPR, which sensitizes the CICR mechanism to Ca\textsuperscript{2+}.

In support of the theory that cADPR amplifies CICR in the heart is the observation above that, if loading of the SR with Ca\textsuperscript{2+} was augmented by exposure to 1 μM ouabain (under conditions in which this concentration was insufficient to initiate oscillatory behavior alone), exogenous cADPR was associated with the development of spontaneous contractile and electrical activity. It therefore seems possible that cADPR may exert an important influence on arrhythmogenic activity in the heart, particularly under conditions where loading of the SR with Ca\textsuperscript{2+} is high, and hence, compounds that reduce the actions of endogenous cADPR may prove useful in the treatment of certain cardiac arrhythmias.