Cyclic adenosine diphosphate ribose (cADPR) is a potent endogenous calcium-mobilizing agent synthesized from β-NAD⁺ by ADP-ribosyl cyclases in sea urchin eggs and in several mammalian cells (Galione, A., and White, A. (1994) Trends Cell Biol. 4, 431-436). Pharmacological studies suggest that cADPR is an endogenous modulator of Ca²⁺-induced Ca²⁺ release mediated by ryanodine-sensitive Ca²⁺ release channels. An unresolved question is whether cADPR can act as a Ca²⁺-mobilizing intracellular messenger. We show that exogenous application of nitric oxide (NO) mobilizes Ca²⁺ from intracellular stores in intact sea urchin eggs and that it releases Ca²⁺ and elevates cADPR levels in egg homogenates. 8-AminocADPR, a selective competitive antagonist of cADPR-mediated Ca²⁺ release, and nicotinamide, an inhibitor of ADP-ribosyl cyclase, inhibit the Ca²⁺-mobilizing actions of NO, while heparin, a competitive antagonist of the inositol 1,4,5-trisphosphate receptor, did not affect NO-induced Ca²⁺ release. Since the Ca²⁺-mobilizing effects of NO can be mimicked by cGMP, they are inhibited by the cGMP-dependent-protein kinase inhibitor, Rp-8-pCPT-cGMPS, and in egg homogenates show a requirement for the guanylyl cyclase substrate, GTP, we suggest a novel action of NO in mobilizing intracellular calcium from microsomal stores via a signaling pathway involving cGMP and cADPR. These results suggest that cADPR has the capacity to act as a Ca²⁺-mobilizing intracellular messenger.

Nitric oxide (NO)¹ is now recognized as a signaling molecule in many mammalian tissues where it has diverse functions as a neurotransmitter as well as an agent mediating apoptosis (1-7). Although NO was first discovered as a mediator of vascular smooth muscle relaxation, where it leads to a decrease in intracellular free calcium [Ca²⁺]i (8), recent reports in intestinal cells in the mammalian gut (9), a macrophage line (10), and pancreatic β cells (11) demonstrate that treatments with NO and NO donors elicit increases in [Ca²⁺]i. These effects persist in the absence of extracellular calcium and can be blocked by pretreatment with ryanodine (9, 11), suggesting that NO may activate a signal transduction cascade, which activates ryanodine-sensitive calcium release channels (RyRs). We have studied this novel aspect of NO action in the sea urchin egg, since Ca²⁺ release mechanisms have been extensively studied in this system (12) and where multiple calcium mobilization pathways have been shown and are amenable to detailed analysis. In the sea urchin egg one Ca²⁺ release mechanism is gated by the established second messenger, inositol 1,4,5-trisphosphate (IP₃), which is produced in response to the interaction of many extracellular stimuli with cell surface receptors (13). Another involves the activation of ryanodine-sensitive calcium release channels (14). RyRs are present on intracellular calcium stores in a wide range of cell types including sea urchin eggs (15). Here ryanodine receptors have been shown to beregulated by cADPR (16), a novel calcium-mobilizing metabolite that is synthesized from β-NAD⁺ by ADP-ribosyl cyclases (12). Accumulating evidence suggests that cADPR is a widespread modulator of ryanodine receptor-mediated calcium release in many different types of mammalian cells (17-26) as well as in plants (27).

A second messenger role for cADPR requires that it mediates the intracellular actions of hormones or neurotransmitters. We show that NO mobilizes calcium from intracellular stores in the sea urchin egg via a pathway in part involving cGMP and leading to the activation of the cADPR-sensitive calcium release mechanism.

**EXPERIMENTAL PROCEDURES**

Collection of Sea Urchin Eggs—Eggs were obtained by stimulating ovulation of female Lytechinus pictus (Marinus, Inc., Long Beach, CA) with an intracoeleomic injection of 0.5 mM KCl solution. These were then washed twice in artificial seawater (435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA at pH 8.0), and jelly was removed by filtration through 85-μm Nitex mesh.

Imaging of Intracellular [Ca²⁺]i—Eggs—Eggs were transferred to poly-L-lysine (10 mg/ml)-coated glass coverslips, allowed to adhere, and microinjected with fura-2, pentapotassium salt (10 mM in the pipette), in buffer consisting of 0.5 M KCl, 20 mM Pipes at pH 6.7 to a final concentration of approximately 10 μM. Injection volumes did not exceed 1% of cell volume. All experiments were performed at 22 °C. Free cytosolic Ca²⁺ concentration was determined by ratiometric fluorescence intensities at excitation wavelengths of 340 and 380 nm, using an emission wavelength of 510 nm. Ratio images were obtained using a fluorimetric imaging system and Invision software supplied by Improvision Ltd., University of Warwick Science Park, Coventry, UK. Standard CaCl₂ solutions were used to calibrate the system, and viscosity corrections were made (28).

NO Applications—NO-containing solutions in respective buffers were prepared by bubbling NO gas (Aldrich) at 4 °C and under oxygen-free conditions to reduce oxidation. The NO concentration was measured with a NO-sensitive electrode (ISO-NO meter, 2-mm diameter electrode; range of 1 nM to 20 μM, World Precision Instruments, St. Petersburg, FL). The electrode was calibrated in accordance with the manu.

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¹ The abbreviations used are NO, nitric oxide; [Ca²⁺]i, intracellular free calcium concentration; CICR, calcium-induced calcium release; cADPR, cyclic ADP-ribose; IM, intracellular medium; IP₃, inositol 1,4,5-trisphosphate; R₈-8-pCPT-cGMPS, Rp isomer of 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphorothioate; RyR, ryanodine receptor; Pipes, 1,4-piperazinediethanesulfonic acid.
Nitric Oxide-induced Ca\(^{2+}\) Mobilization Mediated by cADPR

factor's recommended methodology using a chemical titration method. Known concentrations of KNO\(_2\) are converted to NO in the presence of reducing agents KI and H\(_2\)SO\(_4\). Our stock solutions were \(\sim 350 \mu M\) with respect to gaseous NO. Exposure of eggs to NO was achieved by adding 50 \(\mu l\) of the solution into the imaging chamber (volume, \(\sim 500 \mu l\)) or by the addition of 5–20 \(\mu l\) of the stock solution to the cuvette containing 500 \(\mu l\) of 5% sea urchin egg homogenate in homogenate experiments.

Measurement of cGMP—Cyclic GMP was radioimmunoassayed according to Doshi et al. (29), using a kit supplied by Amershams International plc. Protein was measured by the method of Lowry et al. (30), as modified by Miller (31).

Ca\(^{2+}\) Release Assays—Homogenates (5%) of unfertilized L. pictus eggs (Marinus, Inc.) were prepared as described previously (32). 5% microsomes were purified from homogenates by Percoll centrifugation (33). Ca\(^{2+}\) loading was achieved by incubation at room temperature for 3 h in an intracellular medium (IM) consisting of 250 mM potassium gluconate, 250 mM N-methylglycine, 20 mM Hepes (pH 7.2), 1 mM MgCl\(_2\), 1 mM ATP, 10 mM phosphocreatine, 10 units/ml creatine phosphokinase, 1 \(\mu g/ml\) digoxigenin, 1 \(\mu g/ml\) antimycin, and 3 \(\mu M\) fluo-3. Fluorimetry was performed at 17°C using 500 \(\mu l\) of 5% homogenate, continuously stirred, in a Perkin-Elmer LS-50B fluorimeter. Free Ca\(^{2+}\) concentration was measured by monitoring fluorescence intensity at excitation and emission wavelengths of 490 and 535 nm, respectively. Additions were made in 1–5-\(\mu l\) volumes, and all chemicals were added in IM containing 10 \(\mu M\) EGTA. Basal concentrations of Ca\(^{2+}\) were typically between 100 and 150 nm. Sequestered Ca\(^{2+}\) was determined by monitoring the decrease in fluo-3 fluorescence during microsomal loading and by measuring Ca\(^{2+}\) release in response to ionomycin (5 \(\mu M\)) and was constant between experiments.

cADPR Determinations in Egg Homogenate Treated with NO-containing Solutions—Fluorescent increases obtained in homogenates treated with NO aliquots were translated into cADPR levels from a standard curve of fluorescence versus cADPR concentrations obtained from homogenates to which known amounts of authentic cADPR had been added. The specificity of the bioassay for cADPR (34) was demonstrated by the complete inhibition of NO-induced increases in fluo-3 fluorescence by the prior addition of a desensitizing concentration of cADPR (1 \(\mu M\)) (32) or treatment with the cADPR antagonist, 8-amino-cADPR (35).

Materials—cADPR and 8-amino-cADPR were synthesized as described previously. Ryanodine, fluo-3, and fura-2 were purchased from Calbiochem; R\(_g\)-8-pCPT-cGMP was from Biolog Life Science Institute, Bremen, Germany. All other chemicals were from Sigma.

RESULTS

Fig. 1 shows that in single sea urchin eggs microinjected with the Ca\(^{2+}\) indicator fura-2, application of exogenous NO dissolved in seawater (approximate final concentration of 32 \(\mu M\)) caused an increase in [Ca\(^{2+}\)]. There was a latency of 17 ± 3 s (\(n = 15; S.E.\)) before the initiation of the [Ca\(^{2+}\)] signal, which occurred at a discrete locus and then spread across the egg as a rapid but short-lived Ca\(^{2+}\) wave. The magnitude of NO-induced calcium transients (800 ± 30 nm, \(n = 12; S.E.\)) was generally smaller than those elicited at fertilization (1 ± 0.3 \(\mu M, n = 12; S.E.\)) and did not result in the elevation of fertilization envelopes (0/24 eggs treated with NO, 10–100 \(\mu M\)). Neither the magnitude (780 ± 43 nm, \(n = 10; S.E.\)) nor the latency for the calcium transient (18 ± 4 s, \(n = 10; S.E.\)) was signifi-
cantly affected by the removal of extracellular calcium (Fig. 1, 5th column), indicating that it was produced predominantly by release from intracellular Ca\(^{2+}\) stores.

To confirm that NO was mobilizing Ca\(^{2+}\) from intracellular stores we tested the effects of NO on Ca\(^{2+}\) release in sea urchin egg homogenates. Fig. 2 shows the simultaneous measurement of NO concentration changes and transient Ca\(^{2+}\) release in sea urchin egg homogenates stimulated with a bolus of NO-containing IM solution. There was a rapid increase in NO concentration in the homogenate, which peaked at approxi-
mately 5 \(\mu M\) as measured with a NO electrode that declined over 150 s. The Ca\(^{2+}\) release elicited by this stimulus occurred only after a latency of around 120 s. Fig. 3A shows the effect of varying the concentration of NO (3–10 \(\mu M\)) in the presence of \(\beta\)-NAD\(^+\) (50 \(\mu M\)) and GTP (250 \(\mu M\)). The magnitude of response increased with increasing NO concentrations, whereas the latency was inversely dependent and was as long as 180 s at lower NO concentrations. Using the sea urchin egg microsomes as a bioassay for cADPR, we obtained a concentration-response relationship for NO-induced cADPR production in egg homogenates (Fig. 3A, inset).

The mechanism of NO on Ca\(^{2+}\) release was indirect since it was unable to mobilize Ca\(^{2+}\) from purified microsomes (data not shown), suggesting the requirement for cytosolic factors present in crude homogenate. In addition it also required the presence of \(\beta\)-NAD\(^+\) and GTP. The dependence of both \(\beta\)-NAD\(^+\) and GTP for the Ca\(^{2+}\)-mobilizing effect of NO is shown in Fig. 3B. Addition of NO (9 \(\mu M\)) in the absence of either \(\beta\)-NAD\(^+\) or GTP to egg homogenates alone caused no Ca\(^{2+}\) release. However, Ca\(^{2+}\) release by NO (9 \(\mu M\)) could be reconstituted in the presence of both \(\beta\)-NAD\(^+\) (50 \(\mu M\)) and GTP (250 \(\mu M\)) (Fig. 3B). NO-induced release displayed a latency of \(-100\) s. Increasing the GTP concentration to 500 \(\mu M\) shortened the latency and increased the magnitude of response (Fig. 3B). A rapid Ca\(^{2+}\) release by subsequent addition of cADPR (100 \(nM\)) with no apparent delay could be achieved under all four conditions; however, the magnitude of the cADPR response was diminished in proportion to release obtained with NO. The effects of NO-induced Ca\(^{2+}\) release on the magnitude of subsequent release by either cADPR is shown in Fig. 3C. The more Ca\(^{2+}\) released by NO reduces that triggered by cADPR (100 \(nM\)). The pharmacology of NO-induced Ca\(^{2+}\) release is shown in Fig. 3D. NO-induced Ca\(^{2+}\) release was antagonized by 8-amino-cADPR (400 \(nM\)), which also blocked Ca\(^{2+}\) release by a subsequent
addition of cADPR (200 nM) (Fig. 3D), suggesting that the effect of NO was mediated by cADPR. Consistent with this result was that nicotinamide, which inhibits \( \beta\)-NAD\(^{1}\) conversion to cADPR catalyzed by ADP-ribosyl cyclases,\(^2\) abolished NO but not cADPR-induced Ca\(^{2+}\) release (Fig. 3D). These data suggest that NO-induced calcium release requires the participation of cGMP-dependent protein kinases and ADP-ribosyl cyclases, which may explain the requirement for cytosol as well as GTP and \( \beta\)-NAD\(^{1}\). Since NO required the cGMP precursor GTP for Ca\(^{2+}\) release and an cGMP-dependent protein kinase inhibitor blocked the effects, we examined the effects of cGMP on Ca\(^{2+}\) release in egg homogenates. Previous studies have indicated that cGMP mobilizes Ca\(^{2+}\) in sea urchin eggs (37) and in egg homogenates (38). cGMP alone does not have direct Ca\(^{2+}\) mobilizing activity but has been reported to enhance the synthesis of cADPR from \( \beta\)-NAD\(^{1}\) (38). Fig. 4 shows that in microsomal fractions derived from sea urchin eggs in the presence of 25% supernatant (32, 33), treatment with cGMP leads to the release of calcium from microsomes after a variable latency of a number of seconds. The amplitude of [Ca\(^{2+}\)], release with cGMP was dose-dependent, and the latency in the range of 300–600 s was inversely dependent on cGMP concentration (Fig. 4A). Mobilization of calcium was absolutely dependent on the presence of \( \beta\)-NAD\(^{1}\) (38) and abolished (Fig. 4B) by the competitive cADPR antagonist, 8-amino-cADPR (34). \( R_{p}\)-8-pCPT-cGMPS (200 \( \mu\)M) also completely abolished Ca\(^{2+}\) release by cGMP (data not shown). Heparin (0.2 mg/ml), which blocks IP\(_{3}\) receptors in sea urchin eggs (32, 38) and other tissues, had no inhibitory effect on Ca\(^{2+}\) release in response to cGMP, although it blocked release by IP\(_{3}\) (1 \( \mu\)M) but had no effect on cGMP-induced Ca\(^{2+}\) release. All figures are representative of at least three experiments.

We investigated whether the Ca\(^{2+}\)-mobilizing actions of NO in sea urchin eggs were mediated by cGMP, since NO is well characterized as an activator of soluble guanylate cyclase (7). We measured intracellular levels of cGMP in eggs and egg homogenates treated with NO. In NO-treated eggs there was an approximate doubling in the intracellular levels of cGMP (Fig. 5A). In egg homogenates NO treatments lead to a concentration-dependent increase in the cGMP by over 3-fold (Fig. 5B). To investigate the mechanism of NO-induced Ca\(^{2+}\) mobilization from intracellular stores in intact sea urchin eggs, eggs were treated with pharmacological agents that inhibit NO or cGMP effects or inhibit cADPR-induced Ca\(^{2+}\) release mechanisms (Fig. 6). Eggs microinjected with 8-amino-cADPR to a final concentration of 1 \( \mu\)M (Fig. 6, column 2) showed substantially reduced NO-induced calcium increases in the egg (approximately 90%) compared with the control (Fig. 6, column 1). In experiments in Ca\(^{2+}\)-free medium the response in 8-amino-cADPR-injected eggs was completely abolished (data not shown). \( R_{p}\)-8-pCPT-cGMPS (25 \( \mu\)M) also reduced the NO-induced Ca\(^{2+}\) transient substantially (Fig. 6, column 3), suggesting a role of cGMP and cGMP-dependent protein kinase in mediating Ca\(^{2+}\) mobilization by NO. Hemoglobin that scavenges NO and blocks NO-mediated effects in mammalian systems (2) also blocked the NO-induced Ca\(^{2+}\) signal in sea urchin

\(^2\) J. Sethi and A. Galione, unpublished observations.
enhancement of the response (transient in response to NO (0.4 mg/ml, final concentration) did not reduce the calcium alargeCa2+erasedasarelaxantofsmoothmuscle(8),NOwasfoundtoeliciting pathway. Surprisingly, for an agent that was first discov-
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FIG. 5. NO increases cGMP levels in unfertilized sea urchin eggs and egg homogenates. cGMP levels were measured using a radioimmunoassay protocol (see “Experimental Procedures”). A, nitric oxide was bubbled into degassed artificial seawater, yielding a solution of ~350 µM. 50 µl of this solution was applied to sea urchin eggs (500 µl) suspended in artificial seawater at t = 0 s. Incubations were then continued at 22°C. Levels of cGMP were measured at the times indicated. Results are expressed as the mean of four separate estimations ± S.D. B, cGMP lev-
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DISCUSSION

cADPR has been identified as a potent Ca2+-releasing agent through a Ca2+-release mechanism that is distinct from that regulated by IP3 (32). In many systems, including sea urchin eggs, cADPR appears to act as modulator of CICR through RyRs (41). Although the number of cell types in which cADPR is an effective Ca2+-releasing agent continues to grow, little is known about possible receptor mechanisms that may be coupled to intracellular cADPR production.

In this investigation we identified NO as an agonist that can mobilize intracellular Ca2+ by selectively activating a Ca2+ signaling pathway involving cADPR while having no effect on the IP3 receptor pathway. We have previously shown that cGMP can enhance cADPR synthesis in sea urchin eggs and homogenates (38) and that this may underlie the Ca2+-mobilizing action of cGMP in this cell (37, 42). We have investigated whether the guanylyl cyclase activator NO can also release Ca2+ from intracellular stores by activating the cADPR signaling pathway. Surprisingly, for an agent that was first discovered as a relaxant of smooth muscle (8), NO was found to elicit a large Ca2+ transient in intact sea urchin eggs loaded with the intracellular Ca2+-reporter fura-2 (Fig. 1). The sea urchin egg is rapidly becoming a useful system in which to investigate Ca2+-mobilization since Ca2+ stores in these eggs express multiple Ca2+-release channels that participate in the fertilization Ca2+ wave (43, 44) and the regulation of these channels can be directly investigated in egg homogenates or microsomal preparations (16). The Ca2+-mobilizing action of NO could be re-

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gave rise to regenerative Ca\textsuperscript{2+} waves seen in many single cells and tissues (47). Whether nitric oxide has a role in calcium signaling at fertilization in the sea urchin egg remains to be determined. Since the magnitude of the Ca\textsuperscript{2+} wave elicited by the high concentrations of NO required to induce Ca\textsuperscript{2+} release is insufficient to activate sea urchin eggs, if such a mechanism is employed at fertilization it is likely to be modulatory. One possible role of the NO-activated pathway being investigated is that NO could be locally produced at the site of sperm-egg fusion, which would rapidly diffuse across the entire cell. This could lead to a global rise in cADPR, which could facilitate a wave of CICR across the egg to activate it by sensitizing the egg’s CICR mechanism to activation by increases in Ca\textsuperscript{2+}.

The NO-stimulated Ca\textsuperscript{2+} mobilization pathway involving cADPR/RyRs might augment the recently described effects of NO and cGMP in regulating receptor-mediated Ca\textsuperscript{2+} influx across the plasma membrane in other cells (48), contribute to RyR-based subsarcosomal Ca\textsuperscript{2+} sparks, which have recently been implicated in regulating relaxation of vascular smooth muscle (49), and be important in NO-induced changes in neuronal plasticity (50). Whether the recently described stimulation of ADP-ribosyl cyclases in longitudinal smooth muscle by cholecytokinin (51) involves either NO or cGMP as intermediate remains to be determined.

Fig. 6. Effects of cADPR antagonist (8-amino-cADPR), G-kinase inhibitor (R-8-pCPT-cGMPS), hemoglobin, and heparin on the NO-induced release of intracellular Ca\textsuperscript{2+} in intact sea urchin eggs. Digital ratio images were measured at 22°C, as described in the legend for Fig. 1, and are representative of at least five separate experiments. Column 1, an egg microinjected with fura-2 with a 30 μM NO solution added at t = 0, eliciting a control response. Column 2, as for column 1, except the egg was co-injected with 1 μM 8-amino-cADPR (approximate final concentration). Column 3, as for column 1, except the egg was co-injected with 250 μM (approximate final concentration) R-8-pCPT-cGMPS. Column 4, as for column 1, except 10 mg/ml hemoglobin was added to the bathing medium prior to addition of a NO-containing solution. Column 5, as for column 1, except the egg was co-injected with 400 μg/ml heparin.

Fig. 7. Model for NO-induced Ca\textsuperscript{2+} mobilization in sea urchin eggs. NO activates a soluble guanylate cyclase resulting in the conversion of GTP to cGMP. The resulting cGMP elevation activates a cGMP-dependent protein kinase, which then phosphorylates ADP-ribosyl cyclase or a regulator of the enzyme, catalyzing the conversion of β-NAD\textsuperscript{+} to cADPR. The increase in cADPR levels results in the binding of cADPR to its receptor leading to the opening of a RyR-like Ca\textsuperscript{2+} channel in the endoplasmic reticulum (16), resulting in a rise in [Ca\textsuperscript{2+}]. IP\textsubscript{3}, IP\textsubscript{3} receptor; NiAm, nicotinamide.

Since NO synthesis by constitutive NO synthases is often calcium-dependent (1), a NO-induced rise in [Ca\textsuperscript{2+}] may serve to amplify NO production as previously seen (9) and could also
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