A Novel Adenylyl Cyclase Detected in Rapidly Developing Mutants of Dictyostelium*

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Disruption of either the RDEA or REGA genes leads to rapid development in Dictyostelium. The RDEA gene product displays homology to certain H2-type phospho-

transferases, while REGA encodes a cAMP phosphodiesterase with an associated response regulator. It has been proposed that RDEA activates REGA in a multistep phosphorelay. To test this proposal, we examined cAMP accumulation in rdeA and regA null mutants and found that these mutants show a pronounced accumulation of cAMP at the vegetative stage that is not observed in wild-type cells. This accumulation was due to a novel adenylyl cyclase and not to the known Dictyostelium adenylyl cyclases, aggregation stage adenylyl cyclase (ACA) or germination stage adenylyl cyclase (ACG), since it occurred in an ac&A/rdeA double mutant and, unlike ACG, was inhibited by high osmolarity. The novel adenylyl cyclase was not regulated by G-proteins and was relatively insensitive to stimulation by Mn2+ ions. Addition of the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) permitted detection of the novel adenylyl cyclase activity in lysates of an ac&A/rdeA double mutant. The fact that disruption of the RDEA gene as well as inhibition of the REGA-phosphodiesterase by IBMX permitted detection of the novel AC activity supports the hypothesis that RDEA activates REGA.

Mutants exhibiting an altered rate of development can provide useful insight into rate-limiting events in development (1). There are three classes of rapidly developing mutants in Dictyostelium: rdeA, rdeC, and regA. RdeC mutants lack a functional regulatory subunit of cAMP-dependent protein kinase (PKA), so that PKA activity is constitutive (2). PKA plays an important role in many aspects of metazoan development (3) as well as in learning and memory (4), and genetic manipulation has demonstrated that it is essential for gene expression throughout development in Dictyostelium (see Ref. 5 and references therein). Thus the level of PKA activity appears to have a profound influence on the rate of development in this organism. With regard to the two other classes of rapidly developing mutants, it has been reported that rdeA mutants have elevated intracellular cAMP levels during vegetative growth and possibly during development (6, 7). The RDEA gene product displays some homology to H2-type phosphotransferases in multicomponent signaling systems (8). The C terminus of REGA is a cAMP-phosphodiesterase (cAMP-PDE), while the N-terminal region is homologous to the well-characterized response regulators of bacterial and eukaryotic two-component signaling systems (9–11). It has therefore been proposed that RDEA activates the REGA-PDE in a multistep phosphorelay (8, 11).

We have examined cAMP accumulation in rdeA and regA mutants to explore certain implications of this proposal. During the course of this work we discovered a novel adenylyl cyclase. Until now genes coding for two structurally distinct forms of adenylyl cyclase have been identified in Dictyostelium. The so-called aggregative enzyme (ACA) displays negligible activity in growing (vegetative) cells and accumulates early in development with a maximum at the time of aggregation. Disruption of ACA results in a failure to aggregate (12). ACA activity is stimulated by extracellular cAMP via a G-protein-dependent pathway and rapidly adapts (13). Transcripts of the other cyclase, ACG, are normally detected only during spore germination (12). However, the enzyme can be readily assayed in vegetative cells when expressed under the control of the actin15 promoter. It is strongly stimulated by high osmolarity and functions as an osmosensor controlling spore germination (14). We report the identification of a novel adenylyl cyclase and present evidence for the idea that RDEA activates REGA in a multistep phosphorelay.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—2′-Deoxyadenosine 3′,5′-monophosphate (2′-H-cAMP), guanosine 5′-O-(2-thiodiphosphate) (GDPβS), guanosine 5′-O-(2-thiotriphosphate) (GTPγS), 3-isobutyl-1-methylxanthine (IBMX), dithiothreitol (DTT), and G418 were from Sigma, basicidin was from ICN, and [3H]-cAMP was from Amersham Pharmacia Biotech (Little Chalfont, United Kingdom).

The cell lines acA, acA/ACG (12), DH1/rdeA, AX2/rdeA, acA/ rdeA (8), and AX2/regA (11) were grown in standard axenic medium, which was supplemented with 20 μg/ml G418 for the acA/ACG line. The acA/ACG line harbors a gene fusion of the constitutive actin15 promoter and the entire coding sequence of the ACG gene, causing ACG to be expressed during the entire course of development (12). The acA/ACG line was obtained by transforming the acA line with the ACG gene, in which an internal XbaI-EcoRV fragment was replaced by a basicidin expression cassette (15). Nine basicidin-resistant transformants were selected, and three mutants carrying an acA gene disruption were identified by polymerase chain reaction and Southern blot analysis.

Accumulation of cAMP Accumulation by Intact Cells—Cells were harvested from growth medium, washed with 10 mM sodium/potassium phosphate buffer, pH 6.5, and either resuspended directly in PB at 108 methyloxanthine; PDE, phosphodiesterase; 2′H-cAMP, 2′-deoxyadenosine 3′,5′-monophosphate; GDPβS, guanosine 5′-O-(2-thiodiphosphate); GTPγS, guanosine 5′-O-(2-thiotriphosphate); DTT, dithio- reitol; PB, phosphate buffer; ACB, adenylyl cyclase B.
cells/ml or plated on PB agar at 2.5×10⁶ cells/cm², starved for 6 h at 22 °C, and subsequently collected and resuspended in PB. Cells were stimulated with 5 μM 2′H-cAMP and/or 5 mM DTT (final concentrations), and after variable time periods, the reaction was terminated by adding an equal volume of 3.5% (v/v) perchloric acid. Lysates were neutralized with KHCO₃, and cAMP levels were determined by isotope dilution assay, using purified PKA regulatory subunit (PKA-R) from beef heart as cAMP-binding protein (16).

**In Vitro Adenylyl Cyclase Assays**—Cells were harvested, washed once with PB, resuspended in ice-cold lysis buffer (2 mM MgCl₂ and 250 mM sucrose in 10 mM Tris, pH 8.0), and lysed through nuclepore filters (pore size, 3 μm). Aliquots of 10 μl cell lysate were added to 5 μl of variables and incubated at 0 °C for 5 min. The reaction was started by adding 5 μl of assay mix (2 mM ATP, 40 mM DTT in 2 M 22 °C, H-cAMP and/or 5 mM DTT (final concentrations), and after variable time periods, the reaction was terminated by adding an equal volume of 3.5% (v/v) perchloric acid. Lysates were neutralized with KHCO₃, and cAMP levels were determined by isotope dilution assay, using purified PKA regulatory subunit (PKA-R) from beef heart as cAMP-binding protein (16).

**RESULTS**

**In Vivo cAMP Accumulation in rdeA and regA Mutants**—To investigate cAMP accumulation in rdeA and regA null mutants, we first performed a standard assay for the ACA-mediated cAMP relay response (16). Aggregation-competent cells were stimulated with the cAMP receptor agonist 2′H-cAMP in the presence of DTT, an inhibitor of extracellular PDE (17). Fig. 1A shows that the presence of the rdeA or regA lesions had no pronounced effect on 2′H-cAMP-induced cAMP accumulation. As expected, there was little or no cAMP synthesis in acaA cells (12) or in an acaA/rdeA double mutant. However, when cAMP accumulation was examined in washed vegetative cells, the rdeA and regA mutants both accumulated substantial amounts of cAMP, unlike wild-type (AX2, DH1) or acaA cells (Fig. 1B). Remarkably, this accumulation was also observed in an acaA/rdeA double mutant, indicating that it was not dependent on ACA. In vivo ACG activity is up to 4-fold stimulated by high extracellular cAMP and declined progressively over the first 6 h of starvation. This decline was also evident when cAMP production by vegetative acaA/rdeA cells (Fig. 1B) was compared with that of 6-h starved cells (Fig. 1A).

**Detection and Characterization of the Novel AC Activity in**

![Fig. 1. cAMP accumulation in rdeA and regA mutants. A. cAMP relay response in aggregation competent cells. Parent strains (AX2, acaA) and rdeA and regA mutants were starved for 6 h at 22 °C, resuspended in PB, and stimulated with 5 μM 2′H-cAMP and 5 mM DTT. At the indicated time periods, the reaction was terminated with perchloric acid, and total cAMP levels were measured in the neutralized lysates. Data are standardized on the total protein content of the cell suspension. B. cAMP accumulation by vegetative cells. Parent strains (AX2, DH1, acaA) and rdeA and regA mutants were harvested from growth medium, resuspended in PB, exposed to 5 mM DTT, and assayed for total accumulated cAMP levels at the indicated time periods. C. effects of high osmolarity on cAMP accumulation. Washed vegetative rdeA and regA mutant cells were incubated with 5 mM DTT in the presence of the indicated concentrations of NaCl. After 20 min of incubation, total cAMP levels were determined. Data are expressed as percentage of cAMP levels accumulated in the absence of NaCl. All data represent the means and S.E. of two experiments performed in triplicate. Symbol key: ○, AX2; ●, AX2/rdeA; ▲, AX2/regulated; ○, DH1; ●, DH1/rdeA; ▲, acaA; ■, acaA/rdeA.

![Fig. 2. In vivo adenylyl cyclase activity during starvation of acaA/rdeA cells. acaA/rdeA cells were either used directly from growth medium (t = 0 h), or cells were first plated on PB agar and incubated for 1, 2, 3, 4, 5, or 6 h at 22 °C. Subsequently cells were resuspended in PB to 10⁶ cells/ml, exposed to 5 mM DTT for 0, 2, 5, or 10 min, and assayed for total cAMP levels. Data were standardized on the protein level of the cell suspensions and are expressed as percentage of cAMP accumulated after 10 min in cells at t = 0 h. Means and S.E. of three experiments performed in triplicate are presented.

**Cell Lysates**—ACA activity can be measured in cell lysates provided that cAMP or GTP-γS are present during lysis to activate an associated G-protein (13). ACG activity is not dependent on G-proteins (12). Fig. 3 shows that an activity could be measured in lysates of vegetative acaA/rdeA cells, which presumably corresponded to the activity observed in intact cells. We will further refer to this activity as ACB. It differed from ACA in being neither stimulated by GTP-γS nor inhibited by GDP-βS (Fig. 3A). ACB activity showed a fairly broad pH optimum, while ACA and ACG showed distinct peaks of activity at pH 8.0 and 7.8, respectively (Fig. 3B). In contrast, ACB was clearly distinguishable from ACA and ACG in its response to divalent cations. The catalytic activity of most adenylyl cyclases is stimulated by the binding of divalent cations to a presumed allosteric site (19), and the relative efficacy of Mg²⁺ and Mn²⁺ differs widely between different enzymes. The activity in lysates of vegetative acaA/rdeA cells, due to ACB, is much more efficiently stimulated by Mg²⁺ than by Mn²⁺ (Fig. 3C) and in this respect is reminiscent of the recently described soluble adenylyl cyclase of Sf9 insect ovary cells (20) as well as of the adenylyl cyclase of Escherichia coli (21). In contrast, the activity of ACG (Fig. 3D) and of ACA (12) is more responsive to Mn²⁺, like standard mammalian forms of AC (22) and certain...
other adenylyl cyclases (23–25).

Relationship of ACB to the rdeA and regA Lesions—If the novel activity can be detected in rdeA and regA cells because they lack a cAMP-PDE activity, it should be possible to detect the same activity in the lysates of acaA cells by inhibiting the cAMP-PDE by pharmacological means. To do this we employed IBMX, a well known inhibitor of mammalian cAMP-PDEs (26), and IBMX also allowed us to detect ACB activity in other cell lines that do not carry the ACA or ACG genes. IBMX also allowed us to detect ACB activity in lysates of acaA/rdeA that do not carry the ACA or ACG genes. IBMX was added to the incubation medium containing the cell lysates at the beginning of the incubation period. The presence of IBMX in the incubation medium had no effect on the activity of the enzymes measured in the cell lysates. The data for acaA/ACG, and for ACA in starved wild-type cells stimulated with GTP-S, are retrieved from Schaap et al. (18). Data are expressed as percentage of activity at pH 8.0. C and D, Mg²⁺ and Mn²⁺ dependence of ACB and ACG activity. acaA/rdeA and acaA/ACG cells were lysed in lysozyme buffer containing 0.5 mM MgCl₂. Lysates were incubated with ATP and DTT with the indicated concentrations of Mg²⁺ or Mn²⁺ ions and assayed for cAMP. Data are expressed as percentage of activity obtained at 10 mM Mg²⁺. Data in all panels represent means and S.E. of two experiments performed in triplicate.

FIG. 3. Detection and characterization of ACB activity in cell lysates. A, possible G-protein dependence of ACB activity. Vegetative acaA/rdeA cells were lysed in the presence and absence of 30 µM GTP-S or 30 µM GDPβS. Lysates were incubated at 22 °C with 0.5 mM ATP and 10 mM DTT for the indicated time periods and assayed for cAMP. B, pH dependence of ACB activity. acaA/rdeA cells were lysed in lysis buffer containing 0.5 mM instead of 10 mM Tris-HCl, and lysates were incubated with 0.5 mM ATP and 10 mM DTT in 250 mM potassium phosphate buffer of the indicated pH values. After 10 min of incubation, reactions were terminated and total cAMP levels determined. The data for acaA/ACG, and for ACA in starved wild-type cells stimulated with GTP-S, are retrieved from Schaap et al. (18). Data are expressed as percentage of activity at pH 8.0. C and D, Mg²⁺ and Mn²⁺ dependence of ACB and ACG activity. acaA/rdeA and acaA/ACG cells were lysed in lysozyme buffer containing 0.5 mM MgCl₂. Lysates were incubated with ATP and DTT with the indicated concentrations of Mg²⁺ or Mn²⁺ ions and assayed for cAMP. Data are expressed as percentage of activity obtained at 10 mM Mg²⁺. Data in all panels represent means and S.E. of two experiments performed in triplicate.

The discovery of ACB may help to explain how PKA is activated in Dictyostelium. It has been shown that a number of early and late genes whose expression is dependent upon PKA can nonetheless be expressed (under appropriate conditions) in acaA cells (5, 28–31). Since the PKA regulatory subunit has a very high affinity for cAMP (32), a low level of cAMP production cGMP or cAMP occurred in the presence of added ATP or GTP, respectively, which is possibly due to interconversion of the triphosphates. cAMP accumulation is not affected by Ca²⁺ ions, while cGMP accumulation is completely inhibited. This and the low level of guanylyl cyclase activity in the acaA/ACG lysates make it very unlikely that cAMP synthesis is catalyzed by a guanylyl cyclase.

DISCUSSION

We have detected a novel adenylyl cyclase activity (ACB) in rdeA and regA cells that is distinct from ACA and ACG. Unlike ACA and guanylyl cyclase, ACB is not regulated by G-proteins, and unlike ACG it is not stimulated by high osmolarity. ACB is more effectively stimulated by Mg²⁺ than by Mn²⁺ ions, which is exactly the reverse of ACG, ACA, and guanylyl cyclase (12, 27). The most convincing evidence that ACB is a novel enzyme is the observation that ACB activity can be detected in lysates of the double mutant acaA/ACG in the presence of IBMX.

As noted in the Introduction, it has recently been proposed that the rdeA gene encodes a phosphotransferase that relays a phosphoryl group from an unknown histidine kinase to the response regulator of REGA and in so doing activates the REGA-PDE (8, 11). Since according to that view both rdeA and regA mutants would be expected to lack this cAMP-PDE activity, our finding that ACB can be detected in intact regA and rdeA cells, but not in wild-type cells, is evidence in its favor. This inference is further supported by the finding that the cAMP-PDE inhibitor IBMX, that is known to inhibit the REGA-PDE, permits detection of a similar activity in lysates of the acaA and acaA/ACG cell lines. However a direct demonstration of phosphoryl group transfer from the RDEA to the REGA protein will be necessary before the proposed relay scheme can be accepted with confidence.

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by ACB could account for this even if much of the cAMP produced was degraded by the active REGA-PDE. This argument would appear to apply at least to PKA-dependent gene expression early in development, when we can be confident that ACB is present (Fig. 2). It may also apply later in development, since preliminary data suggest that ACB is present in slugs of wild-type cells. Moreover, as mentioned, a quite low level of adenylyl cyclase activity may be sufficient to activate PKA.

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