Hormonal Control of ADP-ribosyl Cyclase Activity in Pancreatic Acinar Cells from Rats*

Lutz Sternfeld, Elmar Krause, Andreas H. Guse‡, and Irene Schulz‡

From the University of the Saarland, Institute of Physiology, Building 58, 66421 Homburg, Germany and ‡University Hospital Hamburg-Eppendorf, Center for Experimental Medicine, Institute of Biochemistry and Molecular Biology I: Cellular Signal Transduction, Martinistrasse 52, 20246 Hamburg, Germany

Cyclic ADP-ribose, a metabolite of NAD\(^+\) evokes Ca\(^{2+}\) release from intracellular stores in different cells. We have determined the activity of cADPr-producing enzymes (ADP-ribosyl cyclases) in different cellular fractions prepared from isolated pancreatic acinar cells by measuring the conversion of the β-NAD\(^-\) analogs 1,N\(^6\)-etheno-NAD and nicotinamide guanine dinucleotide to the fluorescent products 1,N\(^6\)-etheno-cADPr and cyclic GDP-ribose, respectively. Substrate/product analyses were carried out by reverse-phase high pressure liquid chromatography. In all subcellular fractions examined (cytosol, mitochondria, plasma, and intracellular membranes), ADP-ribosyl cyclase activity was detected except in zymogen granular membranes. Western blot analysis and immunoprecipitation experiments revealed the presence of the ADP-ribosyl cyclase CD38 in both plasma membranes and mitochondria but not in the cytosol. Hormonal stimulation of intact acinar cells for 1 min with acetylcholine (ACh), cholecystokinin (CCK), or bombesin had no effect. The effect of hormonal activation of ADP-ribosyl cyclase was detected following stimulation of the cells with acetylcholine (ACh) or CCK. The Ca\(^{2+}\) wave (7) depends on the secretagogues that is used to maximally stimulate the cell (3, 7, 8), indicating that different extracellular stimuli may utilize a different set of second messengers.

Cyclic adenosine diphosphoribose (cADPr) leads to Ca\(^{2+}\) release from intracellular stores of sea urchin eggs (9) and from different mammalian cell types (reviewed in Ref. 10) including cells from the exocrine (5) and endocrine (11) pancreas. In pancreatic acinar cells, cADPr applied to the cell via a patch pipette induced Ca\(^{2+}\) oscillations (5) and Ca\(^{2+}\) release from intracellular stores when applied to permeabilized cells (12, 13).

Production of cADPr, activated by extracellular signals, has been shown only in a limited number of cell types, e.g. in pancreatic islets (11), smooth muscle cells (14), cardiac myocytes (15), cortical astrocytes (16), and T lymphocytes (17). At present, only limited information is available about hormonal regulation of the enzymes, which can produce cADPr in pancreatic acinar cells. The ectoenzyme CD38 is a candidate (18), and indeed it was shown that following muscarinic stimulation of isolated acini, the cADPr concentration was lower in the homogenate of pancreatic acinar cells from CD38 knockout mice as compared with wild type mice (18). Despite a model consisting of NAD export via connexin 43, extracellular synthesis of cADPr by ecto-CD38, and import of cADPr via CD38 or via nucleoside transporters (20), it remains unclear how an ecto-ADP-ribosyl cyclase such as CD38 can efficiently and rapidly produce an intracellular second messenger. A soluble ADP-ribosyl cyclase was purified from *Aplysia californica* ovotestis (19), and soluble ADP-ribosyl cyclase activities have also been detected in sea-urchin eggs (20), brain homogenates (21), and human T cells (22).

In the present study, we show that in a cytosolic fraction from pancreatic acinar cells, which is devoid of CD38, an increase in both ADP-ribosyl cyclase activity and cGMP accumulation was detected following hormonal stimulation of the cells with acetylcholine (ACh) or cholecystokinin (CCK), whereas bombesin had no effect. The effect of hormonal activation of ADP-ribosyl cyclase could be mimicked by the cGMP analog, dibutyryl-cGMP, indicating involvement of cGMP and a cGMP-dependent kinase in this signaling process.

---

*This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 530, project A5 (to I. S. and E. K.), GU 360/2–4 and 2–5 and SFB 545 (to A. H. G.), Deutscher Akademischer Austauschdienst and Werner-Otto-Stiftung (to A. H. G.), and the Fonds der Chemischen Industrie (14). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  

§To whom correspondence should be addressed. Tel.: 49-6841-1626450; Fax: 49-6841-1626655; E-mail: irene.schulz@uniklinik-saarland.de  

1 The abbreviations used are: IP\(_3\), inositol-1,4,5-trisphosphate; ACh, acetylcholine; BSA, bovine serum albumin; CCK, cholecystokinin; cADPr, cyclic ADP-ribose; eAD, 1,N\(^6\)-ethenoadenosine; eAMP, 1,N\(^6\)-ethenoadenosine-5'-monophosphate; eADPr, 1,N\(^6\)-etheno-ADP-ribose; cADPr, 1,N\(^6\)-etheno-cyclic ADP-ribose; e-NAD\(^+\), 1,N\(^6\)-etheno-NAD\(^+\);

HPLC, high performance liquid chromatography; NGD, nicotinamide guanine dinucleotide; MES, 2-(N-morpholino)ethanesulfonic acid.
Isolation and Fractionation of Pancreatic Acinar Cells—Pancreata from male Wistar rats (140 g) were cleaned, minced to small pieces, and suspended in 5 ml of "buffer A" (130 mM NaCl, 2 mM MgCl₂, 1 mM CaCl₂, 5 mM KCl, 1.2 mM KH₂PO₄, 10 mM glucose, and 20 mM HEPES) supplemented with 0.01% trypsin inhibitor, 0.2% bovine serum albumin (BSA), pH 7.4. Digestion with collagenase (type III; Biochrome Germany) was carried out in two steps with 1,000 units and 1,800 units (BSA), pH 7.4. Digestion with collagenase (type III; Biochrome Germany) was carried out in two steps with 1,000 units and 1,800 units of collagenase for 10 min each at 37 °C under continuous supply of O₂. Cells were washed twice with buffer A and were resuspended in buffer A without BSA supplemented with 0.01% trypsin inhibitor, 0.2% bovine serum albumin (23, 24).

Details of isolation and fractionation of pancreatic acinar cells are given under "Experimental Procedures." The flow diagram (left) illustrates the main centrifugation steps leading to different cellular fractions. Following hormonal stimulation of cells, only two fractions (right) were prepared.

**EXPERIMENTAL PROCEDURES**

**Isolation and Fractionation of Pancreatic Acinar Cells**—Pancreata from male Wistar rats (140 g) were cleaned, minced to small pieces, and suspended in 5 ml of "buffer A" (130 mM NaCl, 2 mM MgCl₂, 1 mM CaCl₂, 5 mM KCl, 1.2 mM KH₂PO₄, 10 mM glucose, and 20 mM HEPES) supplemented with 0.01% trypsin inhibitor, 0.2% bovine serum albumin (BSA), pH 7.4. Digestion with collagenase (type III; Biochrome Germany) was carried out in two steps with 1,000 units and 1,800 units of collagenase for 10 min each at 37 °C under continuous supply of O₂. Cells were washed twice with buffer A and were resuspended in buffer A without BSA supplemented with 0.01% trypsin inhibitor, 0.2% bovine serum albumin (23, 24).

Details for fractionation of pancreatic acinar cells are given under "Experimental Procedures." The flow diagram (left) illustrates the main centrifugation steps leading to different cellular fractions. Following hormonal stimulation of cells, only two fractions (right) were prepared.

**Fig. 1.** Scheme for fractionation of pancreatic acinar cells. Details for fractionation of pancreatic acinar cells are given under "Experimental Procedures." The flow diagram (left) illustrates the main centrifugation steps leading to different cellular fractions. Following hormonal stimulation of cells, only two fractions (right) were prepared.

The preparation of fractions from the cell homogenate led to a protein recovery of 43.1 ± 5.7% (n = 24) from all fractions as compared with the protein in the total homogenate. Protein of the cytosolic fraction contributed with 21.3 ± 2.1%, crude membranes with 10.7 ± 2.0%, zymogen granules with 5.5 ± 0.7%, mitochondria with 2.2 ± 0.7%, and plasma membranes with 3.3 ± 0.6% to total protein of the homogenate. The purity of the fractions was quantified by determination of the relative enrichment of specific markers in the fraction as compared with the homogenate (Table I). Using the Western blot technique with antibodies against the mitochondrial marker, cytochrome C (purified anti-cytochrome C antibody, BD Biosciences, Europe) we found 2.3-fold enrichments in the mitochondrial fraction. The zymogen granular marker amylase (anti-amylase; Calbiochem) was 2.1-fold enriched in the zymogen granular fraction. Enzymatic detection of the magnesium-dependent Na/K-ATPase (25) (plasma membrane marker) showed a 3.8-fold enrichment.

For measurement of hormone-stimulated cytosolic ADP-ribosyl cyclase activities, it was necessary to shorten the protocol, since hormone-stimulated enzyme activities decreased rapidly during the fractionation procedure. Following the centrifugation step at 1,000 x g for 5 min, the pellet was discarded. The supernatant containing cell membranes and mitochondria was centrifuged immediately thereafter at 100,000 x g for 60 min. The resulting pellet was defined as "membranes" and the supernatant as "cytosol." Marker enzymes for other organelles were

---

**Table I.**

| Fraction          | Protein Recovery (%) |
|-------------------|----------------------|
| Cytosol           | 43.1 ± 5.7%          |
| Crude membranes   | 21.3 ± 2.1%          |
| Zymogen granules  | 10.7 ± 2.0%          |
| Mitochondria      | 5.5 ± 0.7%           |
| Plasma membranes  | 2.2 ± 0.7%           |
| Total homogenate  | 100%                 |
ADP-ribosyl Cyclase Activity in Pancreatic Acinar Cells

Relative enrichment of marker enzymes in cellular fractions as compared with the total homogenate

| Enzyme         | Homogenate | Cytosol* | Plasma membranes | Crude membranes | Zymogen granules | Mitochondria |
|----------------|------------|----------|------------------|-----------------|-----------------|--------------|
| Na/K-ATPase    | 1          | ND       | 3.8 ± 0.8         | 0.8 ± 0.2       | 0.2 ± 0.1       | 0.4 ± 0.3    |
| Cytochrome c   | 1          | ND       | 0.3 ± 0.1         | 0.4 ± 0.1       | <0.1 ± 0.1      | 2.3 ± 0.2    |
| Amylase        | 1          | 0.6 ± 0.3 | 0.3 ± 0.1         | 0.6 ± 0.9       | 2.1 ± 0.3       | 0.3 ± 0.1    |
| CD38           | 1          | ND       | 2.4 ± 0.5         | 1.3 ± 0.1       | <0.1 ± 0.1      | 1.2 ± 0.1    |

apparently absent in the “cytosol” of this preparation, similar to the findings shown in Table I.

Isolation of Zymogen Granular Membranes from the Zymogen Granule Fraction—Purified “zymogen granular membranes” were obtained by suspending “zymogen granules” in 10 ml of “lysis buffer” (in 0.1 M MgSO₄, 5 mM EDTA, 10 mM HEPES, adjusted to pH 7.0 with Tris, supplemented with 10 μg/ml leupeptin, 1 mM benzamidine, and 50 μg/ml trypsin inhibitor) and were kept on ice for 30 min. The resulting suspension was centrifuged at 100,000 × g for 60 min, and the pellet was resuspended in buffer A without BSA (28).

Assay for ADP-ribosyl Cyclase Activity—ADP-ribosyl cyclase activity was measured fluorometrically in subcellular fractions (27). The fractions were adjusted to a protein concentration of 1 mg/ml in buffer A, and 0.1 M LAV-etheno-NAD⁻ (e-NAD⁻) was added at continuous shaking at 37 °C. Enzymatic degradation of e-NAD⁻ to e-ADP, e-ADPr, and other metabolites was accompanied by an approximately 10-fold fluorescence increase and was monitored continuously at 410 nm (emission wavelength) and at an excitation wavelength of 300 nm, using a Spectro Fluoromax fluorometer.

Precise analysis of products of e-NAD⁺ metabolism was carried out by reverse-phase HPLC. Enzymatic degradation of e-NAD⁺ was stopped by cooling the sample rapidly to 4 °C and subsequent filtration at 13,000 × g for 30–60 min through a 10-kDa cut-off “Microcon Centrifugation Filter” (Millipore). The duration of the enzyme assay was adjusted to obtain linear initial velocities according to the ADP-ribosyl cyclase activities present in each fraction (e.g. homogenate and membrane fractions 10 min; cytosolic fraction 60 min).

Different substrate concentrations (10–500 μM) did not change the substrate/product ratio. Desalting of the cytosolic fraction for removal of endogenous NAD⁺ did not have any significant influence on the specific ADP-ribosyl cyclase activity and was therefore not applied routinely. In several experiments, the pseudosubstrate NGD (100 μM) was used instead of e-NAD⁺; however, the specific product generated by the ADP-ribosyl cyclase is cyclic GMP, which was separated and quantified by HPLC.

HPLC Analysis of e-labeled Adenosine Nucleotides—For identification and quantification of different e-adenosine nucleotides that can be generated by NADases (27), separation of the products was necessary. This was performed by HPLC techniques (28) similar to that used to measure endogenous cADPr (29). Samples were immediately analyzed by HPLC or stored at −70 °C for later analysis. For identification of e-ADPr, 200 μl of the filtrate was injected onto a reverse-phase HPLC column (Hypersil BDS C18 column, 5 μm; 250 × 4.6 mm). A gradient of 0.1 M KH₂PO₄, 5 mM tetrabutylammonium hydrogen phosphate, pH 6.0) to methanol was used at a flow rate of 1 ml/min (28, 29). Methanol concentrations in the gradient were 5% at time 0; 7.5% at 2.5 min; 16% at 4–5 min; 25% at 6.5–17 min; and 5% at 18 min. LAV-Etheno-labeled adenosine nucleotides were detected with a fluorescence detector (410-nm emission and 300-nm excitation wavelength). Identification and quantification of the e-adenosine-labeled nucleotides were performed by comparing the retention time and peak of our samples with those of known standards. The standards e-NAD⁺, e-ADP, e-AMP, and e-ADPr were commercially available. e-ADPr was generated by incubating e-NAD⁺ (100 μM) with Aplysia californica ADP-ribosyl cyclase (100 units/ml) for 5 min. e-ADPr was generated from e-ADPr by heating to 80 °C for 60 min (28, 29).

The minimum detection limits of the products in HPLC separation (defined as a 3:1 signal/noise ratio) were 0.85 ± 0.15 pmol for e-ADPr and 3.1 ± 0.48 pmol for e-ADPr.

Western Blotting and Immunoprecipitation of CD38—For Western blot analysis, proteins were separated by SDS-PAGE (12.5% acrylamide) in Laemmli minigels and transferred onto nitrocellulose membranes. Blots were blocked for 1 h with 1% BSA and incubated with a CD38 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and were visualized using the enhanced chemiluminescence technique.

For immunoprecipitation of CD38, 500 μl of cell lysate (adjusted to 1 mg/ml protein with buffer B (10 mM Tris, 1 mM diithiothreitol, 5 mM EGTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 0.50% Triton X-100, 0.2 mg phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin)) was incubated with a 4-μl stock solution of a CD38 antibody (200 μg/ml; AT-1 antibody; Sigma) for 90 min at 4 °C. After the addition of 12 μl of protein G-protein A-Sepharose beads (Oncogene), incubation was continued for 90 min. Subsequently, beads were washed twice with buffer B and finally with buffer B free of Triton X-100. For SDS-PAGE, the pellets were resuspended in 30 μl of SDS-loading buffer (10 mM Tris, 1% mercaptoethanol, 1% SDS, 10% glycerol, and 0.015% bromphenol blue) and heated for 5 min at 95 °C. For measurement of ADP-ribosyl cyclase activity, the pellet was resuspended in 500 μl of buffer A, and the activity was measured as described above.

Determination of cGMP Concentrations in Intact Pancreatic Acinar Cells—Cyclic GMP concentrations were determined using a commercially available enzyme immunoassay (Amersham Biosciences RPN 226). Freshly prepared pancreatic acinar cells (2 mg of protein/ml) were suspended in 200 μl of buffer A and incubated for 1 min in the presence of the indicated hormone or of its carrier (control). After centrifugation (2 min, 250 × g at 4 °C) and removal of the supernatant, intracellular cGMP concentrations were determined according to the manufacturer’s instructions.

RESULTS

Assay for Quantification of ADP-ribosyl Cyclase Activities

To identify ADP-ribosyl cyclase activities in different cellular fractions and organelles we used e-NAD⁺ as substrate. Cleavage of the nicotinamide moiety by enzymatic metabolism of e-NAD⁺ to nucleotides such as e-ADP, e-ADPr, e-cADPr, and e-ADPr resulted in an ~10-fold increase in fluorescence intensity (27). Commercially available ADP-ribosyl cyclase (Aplysia californica, 25 units) converted all e-NAD⁺ (100 μM) within the first 2 min, whereas different subcellular fractions purified from pancreatic acinar cells showed less pronounced enzyme activities (data not shown). Ectocellular ADP-ribosyl cyclase and/or NADase activities in intact pancreatic acinar cells were detected when e-NAD⁺ was present in the extracellular buffer (data not shown). No NADase activity was detected after heat inactivation of proteins (data not shown).

In order to determine the specific ADP-ribosyl cyclase activity out of the whole NAD⁺-metabolizing activities, it was necessary to separate the e-products by HPLC. Fig. 2 shows a typical HPLC separation of e-adenine nucleotides, which were generated by endogenous enzymatic activity in different pancreatic acinar cell fractions. For comparison, separation of standard e-adenine nucleotides is shown. All fractions were incubated with the same amount of e-NAD⁺.

In all fractions, the substrate was converted to different e-adenine nucleotides, indicating that several pathways for NAD⁺ metabolism are present in pancreatic acinar cells. In all fractions, except the zymogen granules, e-ADPr was produced although at varying amounts and specific activities. To exclude the possibility that the substrate e-NAD⁺ behaves different as
Pancreatic acinar cells were isolated and fractionated, and ADP-ribosyl cyclase activities were measured as described under “Experimental Procedures.” Enzymatic reactions were performed at 37°C and stopped after 60 min for the “cytosolic” and “zymogen granular” fraction or after 10 min for the other fractions. The amount of e-cADPr was quantified by HPLC and is presented as the specific activity (nmol of e-cADPr/μg of protein × min) (n = 4–19) or as the relative amount in the respective fraction as compared with the overall activity in the homogenate (% of homogenate).

| Subcellular fraction          | ADP-ribosyl cyclase activity | Relative ADP-ribosyl cyclase activity |
|------------------------------|-----------------------------|--------------------------------------|
| Homogenate                   | 0.76 ± 0.09                 | 100                                  |
| Mitochondria                 | 1.12 ± 0.11                 | 21.2 ± 2.6                           |
| Plasma membranes             | 0.82 ± 0.10                 | 12.8 ± 1.9                           |
| Crude membranes              | 0.51 ± 0.10                 | 15.7 ± 5.2                           |
| Cytosol                      | 0.20 ± 0.06                 | 26.4 ± 4.8                           |
| Zymogen granules             | 0.06 ± 0.01                 | 1.8 ± 0.4                            |
| Zymogen granular membranes   | 0.03 ± 0.01                 | <0.1                                 |

This indicates that a significant part of total enzymatic activity is present in the cytosol to allow for substantial and rapid cADPr production following hormonal stimulation.

Identification of CD38 in Pancreatic Acinar Cells

It is known that pancreatic acinar cells express the ectoenzyme CD38, which combines ADP-ribosyl cyclase, cADPr-hydrolase, and NAD-hydrolase activities (18). As shown in Fig. 3, the antibody specific for human CD38 recognized a protein with the expected molecular mass of CD38 (42 kDa) in the plasma membrane fraction, the crude membranes, and mitochondrial fractions. Furthermore, a weak signal was present in zymogen granules. However, in zymogen granular membranes as well as in the cytosolic fraction, CD38 was not detected, even if the amount of protein applied to the SDS-gel was increased 10-fold (data not shown).

To further prove the absence of CD38 from the cytosol, we concentrated and purified CD38 by immunoprecipitation and Western blot analysis. As shown in Fig. 4A, the anti-CD38 antibody AT-1 precipitated a 42-kDa protein only from the membranes and not from the cytosolic fraction.

Since measurement of enzymatic activities might be more sensitive than Western blot analysis, we measured the ADP-ribosyl cyclase activity in both the precipitate and the supernatant of the first washing step. The precipitate from the membranes showed an ADP-ribosyl cyclase activity of 25 ± 5.6% of total activity (Fig. 4B), whereas 57 ± 8.9% remained in the supernatant. For the cytosol, 95 ± 7.9% remained in the supernatant, and no ADP-ribosyl cyclase activity was detected in the precipitate (Fig. 4B). This confirms the Western blot data (Fig. 3) and suggests that the ADP-ribosyl cyclase activity found in the cytosol is different from CD38.

Furthermore, the AT-1 antibody (5 μg/ml) partially inhibited ADP-ribosyl cyclase activity in the membranes but failed to alter the cytosolic ADP-ribosyl cyclase activity (Fig. 4B). Heat inactivation (15 min, 80°C) resulted in a complete loss of ADP-ribosyl cyclase activity (Fig. 4B), indicating that cADPr was indeed produced by enzymatic activity.

All together, we provide evidence that CD38 is the ADP-ribosyl cyclase activity found in plasma membranes and mitochondria, whereas the cytosolic ADP-ribosyl cyclase activity is an enzyme immunologically different from CD38.

Hormonal Regulation of ADP-ribosyl Cyclases

Ectocellular ADP-ribosyl Cyclase in Pancreatic Acinar Cells—To test whether ecto-CD38 might be hormonally regu-
activity is not involved in hormonally regulated cADPr production.

**Hormonal Activation of Cytosolic ADP-ribosyl Cyclase**—Stimulation of intact acinar cells for 0.5–5 min with ACh or CCK and subsequent fractionation showed increased ADP-ribosyl cyclase activity in the cytosolic fraction. Maximal stimulation was detected between 1 and 5 min. In contrast to ACh and CCK, bombesin had no effect on the ADP-ribosyl cyclase activity in any fraction (Fig. 5A).

Whereas concentrations of CCK below 0.1 nM did not increase ADP-ribosyl cyclase activity as compared with unstimulated cells, enzyme activity was enhanced by CCK concentrations of 1 nM to 121.1 ± 12.7%, of 10 nM to 170.6 ± 11.2%, and of 100 nM to 168.6 ± 15.6%. The maximal effect of ACh was found at 1 μM ACh (151.2 ± 11.1%). Higher or lower concentrations led to a decreased activity of ADP-ribosyl cyclase in the cytosol (Fig. 5B). **Identification of a Pathway That Stimulates ADP-ribosyl Cyclase Activity in Pancreatic Acinar Cells**

In order to investigate possible mechanisms involved in the activation of ADP-ribosyl cyclases, ADP-ribosyl cyclase activities in membranes and the cytosol following stimulation of intact cells with membrane-permeant analogs of cyclic nucleotides were determined. Incubation of intact cells with 1 mM dibutyryl-cGMP led to a 1.9 ± 0.4-fold stimulation of cytosolic ADP-ribosyl cyclase activity compared with control cells, whereas the activity in the membrane fraction remained unchanged (1.03 ± 0.25-fold) (Fig. 6). Since stimulation of intact cells with 1 mM dibutyryl-cAMP did not alter ADP-ribosyl cyclase activity, nonspecific effects of dibutyryl compounds can be excluded (Fig. 6). Interestingly, cGMP added to separated subcellular fractions of pancreatic acinar cells failed to alter the ADP-ribosyl cyclase activity, indicating that additional components are necessary for ADP-ribosyl cyclase activation. Since cGMP is generated in pancreatic acinar cells following hormonal stimulation (33), we tested whether cGMP is produced by the same hormones that also activate the cytosolic ADP-ribosyl cyclase in our system within the short time of stimulation used here (i.e. 1 min). Using an enzyme immunoassay for cGMP, we found that stimulation for 1 min of intact pancreatic acinar cells by CCK (10 nM) or ACh (1 μM) led to a rise in the cellular cGMP concentration of 110 ± 6 (control) to 210 ± 14 and to 187 ± 7 fmol of cGMP/mg of protein, respectively. Bombesin (10 nM) led to a very small rise in cGMP production (125 ± 9 fmol of cGMP/mg of protein) (Fig. 5C).

**DISCUSSION**

**Hormonal Production of cADPr**—The data presented here demonstrate (i) that ADP-ribosyl cyclase activity can be detected by a sensitive HPLC method in the cytosol, mitochondria, and plasma membranes of pancreatic acinar cells; (ii) that mitochondria and plasma membranes, but neither the cytosol nor zymogen granules contain CD38; (iii) that only the cytosolic ADP-ribosyl cyclase, which is different from CD38, can be stimulated by CCK and ACh; (iv) that cGMP is synthesized upon CCK and ACh stimulation in intact cells; and (v) that membrane-permeant dibutyryl-cGMP produces a similar activation of cytosolic ADP-ribosyl cyclase as compared with hormonal stimulation.

Receptor-mediated stimulation of ADP-ribosyl cyclase has also been shown for other cell types such as human T-lymphocytes (22), neonatal rat cardiac myocytes triggered by isoproterenol or angiotensin II (15, 34), and rat cortical astrocytes stimulated by isoproterenol (16).

So far, in none of these systems could the ADP-ribosyl cyclase be identified. To date, the only mammalian ADP-ribosyl
cyclases identified on the molecular level are the ectoenzymes CD38 and CD157 (35). These multifunctional enzymes produce mainly ADPr and only small amounts of cADPr (usually between 1 and 10% of the metabolized substrate) (36).

The similarity of the product pattern of ADP-ribosyl cyclase (see Fig. 2) from the cytosolic fraction (free of CD38) as compared with that of the plasma membrane fraction, which does contain CD38 (see Fig. 3), indicates enzymological similarities between both enzymes. A similar product pattern was also obtained from crude membranes and cytosolic fractions of Jurkat T cells (28, 37). However, our findings that hormones stimulate cytosolic enzyme exclusively indicate fundamental differences between the cytosolic enzyme and CD38. Furthermore, immunological differences between both enzymes were revealed by Western blot analysis (see Fig. 3) and by the inhibitory effect of the anti-CD38 antibody on ADP-ribosyl

---

**Fig. 5.** Hormonal stimulation of ADP-ribosyl cyclases in rat pancreatic acinar cells. A, ADP-ribosyl cyclase activity was measured in the cellular homogenate (n = 12), membranes (n = 12), and cytosol (n = 23) obtained by rapid fractionation (see “Experimental Procedures” and Fig. 1) of intact pancreatic acinar cells that had been incubated in the presence or absence of the indicated hormones for 1 min. The fractions at protein concentrations of 1 mg/ml were equilibrated in buffer A, and the enzyme assay was started by adding 100 μM ε-NAD⁺. The amount of generated ε-cADPr was detected by HPLC separation and presented as percentage of ε-cADPr produced as compared with the ε-cADPr production in cell fractions from unstimulated cells (control = 100%, n = 3–19). For measurement of ectocellular enzyme activities in the supernatant from intact cells, 100 μM ε-NAD⁺ was added to cells followed by the addition of the indicated hormones 5 min later. After 5 min of incubation with the hormones, the supernatant of the intact cells was collected, and the amount of ε-cADPr was quantified by HPLC. It is presented as percentage of ε-cADPr of the ε-cADPr production in the supernatant from unstimulated intact cells (control = 100%, n = 5). B, ADP-ribosyl cyclase activity in the cytosolic fraction following stimulation of intact cells at different hormone concentrations. The amount of generated ε-cADPr was quantified by HPLC separation and is presented as percentage of ε-cADPr produced as compared with the ε-cADPr production in unstimulated cell fractions (control = 100%). C, production of cGMP in intact acinar cells. Freshly prepared pancreatic acinar cells were incubated with the indicated hormone or its carrier for 1 min. After lysis of the cells, intracellular cGMP concentrations were determined by an enzyme immunoassay (see “Experimental Procedures”). Statistical analysis was performed using Student’s t test for paired values. Mean values ± S.E. are compared for unstimulated (control) and stimulated cells.
ADP-ribosyl Cyclase Activity in Pancreatic Acinar Cells

FIG. 6. Stimulation of ADP-ribosyl cyclases by cyclic nucleotides in rat pancreatic acinar cells. ADP-ribosyl cyclase activity was measured in membranes and cytosol obtained by rapid fractionation (see "Experimental Procedures" and Fig. 1) with or without previous stimulation of intact pancreatic acinar cells in the presence of the membrane-permeant analogs of cGMP or cAMP (dibutyryl-cGMP (1 mM, n = 5) and dibutyryl-cAMP (1 mM, n = 6)) for 5 min. For determination of the specific ADP-ribosyl cyclase activities, the fractions (1 mg of protein/ml) were equilibrated in buffer A, and the enzyme assay was started by adding 100 µM ε-NAD⁺. The amount of generated ε-cADPr was quantified by HPLC separation and presented as percentage of ε-cADPr produced as compared with the ε-cADPr production in unstimulated cell fractions (control = 100%). Statistical analysis was performed using Student's paired t test. Mean values ± S.E. are compared from unstimulated (control) and stimulated cells.

cyclase activity in the CD38-positive membrane fraction but not on that in the cytosol (see Fig. 4). The differences in the results from experiments on hormonal stimulation, Western blot analysis, and immunoprecipitation (see Figs. 3–5) indicate that the cytosolic ADP-ribosyl cyclase is a novel, yet unidentified, mammalian ADP-ribosyl cyclase, different from CD38.

Our finding that hormonal stimulation that leads to cytosolic Ca²⁺ concentrations of >100 nM also increases the activity of a cytosolic ADP-ribosyl cyclase extends a model suggested by De Flora et al. (reviewed in Ref. 38). This model assumes that NAD⁺ leaves the cytoplasmic compartment by the specific transport system connexin 43 hemichannels only at cytosolic Ca²⁺ concentrations of <100 nM (39). Then NAD⁺ is converted extracellularly to cADPr by the ectoenzyme CD38 and subsequently is transported back into the cytosol by CD38 and/or an additional specific transport system (16, 39–42). Experimental evidence for this model has been accumulated mainly in HeLa and 3T3 cells transfected with CD38 (39–41, 43); however, because connexin 43 hemichannels are in a closed state at cytosolic Ca²⁺ concentrations of >100 nM, the significance of this model is limited to the regulation of the basal cADPr concentration in cells.

Our data, which suggest that in pancreatic acinar cells the ADP-ribosyl cyclase CD38 is not involved in the hormonal induced signal pathway, are in contrast to data recently published by Fukushi et al. (18). These authors found that stimulation of pancreatic acinar cells by ACh increased the cellular content of cADPr in cells from wild type mice but not in those from CD38 knockout mice. Although the reason for this discrepancy is unclear, it might well be due to differences in animal species (e.g. rats versus mice) or due to the methods used. In our study, the enzymatic activity of ADP-ribosyl cyclase was measured, whereas Fukushi et al. (18) determined the cellular concentration of endogenously produced cADPr. Both an increased production rate of cADPr and a decreased rate of hydrolysis might result in higher cADPr levels. We can not completely exclude hormonal induced inhibition of the rate of hydrolysis of cADPr. The latter would mimic the stimulatory effect of ADP-ribosyl cyclase activity. However, we assume that inhibition of a cADPr-hydrolase at unchanged cyclase activity would change the ratio of cADPr/ADPr, since cADPr would increase and ADPr (the hydrolase product) should decrease. Comparison of the ratio of both enzymatic products under resting conditions as well as at stimulation resulted in no significant change in the product ratio. Therefore, an activation of an unknown hydrolase is unlikely to be responsible for an increased cADPr production.

Physiological Significance of cADPr Production in Pancreatic Acinar Cells—Using video imaging, measurements of [Ca²⁺]ᵢ intracellular stores from pancreatic acinar cells directly showed that cADPr induces Ca²⁺ release from stores located in the basolateral cell side but not from apically located Ca²⁺ stores (13). In contrast, IP₃ induced Ca²⁺ release from all parts of a cell, with highest sensitivity in the apical region (13, 44). This arrangement of IP₃- and cADPr-sensitive Ca²⁺ pools in combination with the data described here could explain why the speed of a Ca²⁺ wave from the apical to the basolateral pole differs when different hormones were used to induce Ca²⁺ release (3, 7, 8, 44). Since stimulation with either ACh or CCK, but not with bombesin, led to activation of cytosolic ADP-ribosyl cyclase (data of the present study), the higher speed of Ca²⁺ waves seen with CCK and ACh than with bombesin (3, 8) could be also due to CCK- or ACh-stimulated production of cADPr and subsequent acceleration of Ca²⁺-induced Ca²⁺ release. Since in the present study hormonal stimulated ADP-ribosyl cyclase activity was found for the first time in the cytosol of acinar cells, the question remains open of how coupling of this enzyme to the hormone receptor might occur. It is unlikely that a translocation of the enzyme from the cytosol to the plasma membrane occurs during hormonal stimulation, since hormone-induced changes in enzymatic activities were not found in plasma membranes. Another possibility for a link between hormone receptor and cytosolic ADP-ribosyl cyclase could be generation of a cytosolic signal such as by an NO/cGMP-activated pathway (45). However, inhibition of ADP-ribosyl cyclase by NO and a concentration-dependent decrease in cytosolic [Ca²⁺] was also described in cultured coronary artery smooth muscle cells (46).

Our finding that dibutyryl-cGMP stimulates ADP-ribosyl cyclase activity and that hormones that activate ADP-ribosyl cyclase activity also increase endogenous cGMP production (see Figs. 5 and 6) indicates that cGMP could be a potential messenger for the mechanism by which hormones activate the cytosolic ADP-ribosyl cyclase.

In conclusion, our data strongly suggest that a cytosolic ADP-ribosyl cyclase immunologically distinct from CD38 is stimulated by CCK or ACh to catalyze cGMP-mediated formation of cADPr with a consequent Ca²⁺ release from rat pancreatic acinar cells.

REFERENCES

1. Streb, H., Irvine, R. F., Berridge, M. J., and Schulz, I. (1983) Nature 306, 67–69
2. Nathansohn, M. H., Padfield, P. J., O'Sullivan, A. J., Burgstahler, A. D., and Jamieson, J. D. (1992) J. Biol. Chem. 267, 18118–18121
3. Pfeiffer, F., Sternfeld, L., Schmid, A., and Schulz, I. (1998) Am. J. Physiol. C063–C072
4. Kasi, H., and Augustine, G. J. (1990) Nature 348, 735–738
5. Thorn, F., Gerasimenko, O., and Petersen, O. H. (1994) EMBO J. 13, 2038–2043
6. Watkin, M., Oaschup, Y. V., and Petersen, O. H. (1990) Cell 63, 1025–1032
7. Xu, X., Zeng, W., Diaz, J., and Mualem, S. (1996) J. Biol. Chem. 271, 24684–24690
8. Gonzalez, A., Schmid, A., Sternfeld, L., Krause, K., Salido, G. M., and Schulz, I. (1999) Biochim. Biophys. Res. Commun. 261, 726–733
9. Lee, H. C., Walsch, T. F., Bratt, G. T., Hayes, R. N., and Clapper, D. L. (1989) J. Biol. Chem. 264, 1032–1038
10. Lee, M. G., Xu, X., Zeng, W., Diaz, J., Wojcikiewicz, R. J., Kuo, T. H., Wuytack, F., Racymaekers, L., and Muallem, S. (1997) J. Biol. Chem. 272, 15765–15770
11. Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993) Science 259, 370–373
12. Gohel, A., Krause, E., Feick, P., and Schulz, I. (2001) Cell Calcium 29, 29–37
13. Krause, E., Gohel, A., and Schulz, I. (2002) J. Biol. Chem. 277, 11696–11702
14. Kuenmerle, J. F., and Makhlouf, G. M. (1995) J. Biol. Chem. 270,
ADP-ribosyl Cyclase Activity in Pancreatic Acinar Cells

15. Higashida, H., Egorova, A., Higashida, C., Zhong, Z. G., Yokoyama, S., Noda, M., and Zhang, J. S. (1999) J. Biol. Chem. 274, 33348–33354
16. Hotta, T., Asai, K., Fujita, K., Kato, T., and Higashida, H. (2000) J. Neurochem. 74, 669–675
17. Guse, A. H., Roth, E., and Emmrich, F. (1994) Biochem. J. 301, 83–88
18. Fukushima, Y., Kato, I., Takasawa, S., Sasaki, T., Ono, B. H., Sato, M., Ohsaga, A., Sato, K., Shirato, K., Okamoto, H., and Maruyama, Y. (2001) J. Biol. Chem. 276, 649–655
19. Hellmich, M. R., and Strumwasser, F. (1991) Cell Regul. 2, 193–202
20. Graeff, R. M., Franco, L., De Flora, A., and Lee, H. C. (1998) J. Biol. Chem. 273, 118–125
21. Matsuzuma, N., and Tanuma, S. (1998) Biochem. Biophys. Res. Commun. 253, 246–252
22. Guse, A. H., da Silva, C. P., Berg, I., Skapenko, A. L., Weber, K., Heyer, P., Hohenegger, M., Ashamu, G. A., Schulze-Koops, H., Potter, B. V., and Mayr, G. W. (1999) Nature 398, 70–73
23. De Lisle, R. C., Schulz, I., Tyrakowski, T., Haase, W., and Hopfer, U. (1984) Am. J. Physiol. 246, G411–G418
24. Lopez-Mediavilla, C., Orfao, A., San Miguel, J., and Medina, J. M. (1992) Exp. Cell Res. 203, 134–140
25. Scharschmidt, B. F., Keeffe, E. B., Blankenship, N. M., and Ockner, R. K. (1979) J. Lab. Clin. Med. 93, 790–799
26. Thevenod, F., Hildebrandt, J. P., Striessnig, J., de Jonge, H. R., and Schulz, I. (1996) J. Biol. Chem. 271, 3300–3305
27. Kleb, B. M., and Pette, D. (1996) Anal. Biochem. 239, 145–152
28. Schweitzer, K., Mayr, G. W., and Guse, A. H. (2001) Anal. Biochem. 299, 218–236
29. da Silva, C. P., Potter, B. V., Mayr, G. W., and Guse, A. H. (1998) J. Chromatogr. B Biomed. Sci. Appl. 707, 43–50
30. Graeff, R. M., Walseh, T. F., Fryxell, K., Branton, W. D., and Lee, H. C. (1994) J. Biol. Chem. 269, 30260–30267
31. Muller-Steinfurter, H., Augustin, A., and Schuber, F. (1997) Adv. Exp. Med. Biol. 419, 389–409
32. Meszaros, L. G., Wrenn, R. W., and Varadi, G. (1997) Biochem. Biophys. Res. Commun. 234, 252–256
33. May, R. J., Conlon, T. P., Erspamer, V., and Gardner, J. D. (1978) Am. J. Physiol. 235, E112–E118
34. Higashida, H., Zhang, J., Hashii, M., Shintaku, M., Higashida, C., and Takeda, Y. (2000) Biochem. J. 352, 197–202
35. Lee, H. C. (2001) Annu. Rev. Pharmacol. Toxicol. 41, 317–345
36. Lee, H. C. (2000) J. Membr. Biol. 173, 1–8
37. De Flora, A., Guida, L., Franco, L., Zocchi, E., Bruzzone, S., Benatti, U., Damonte, G., and Lee, H. C. (1997) J. Biol. Chem. 272, 12945–12951
38. De Flora, A., Guida, L., Franco, L., Bruzzone, S., and Zocchi, E. (2002) (Lee, H. C., ed) pp. 234–268, Kluwer Academic Publishers, Boston
39. Bruzzone, S., Guida, L., Zocchi, E., Franco, L., and De Flora, A. (2001) FASEB J. 15, 10–12
40. Zocchi, E., Daga, A., Usai, C., Franco, L., Guida, L., Bruzzone, S., Costa, A., Marchetti, C., and De Flora, A. (1996) J. Biol. Chem. 271, 8017–8024
41. Franco, L., Guida, L., Bruzzone, S., Zocchi, E., Usai, C., and De Flora, A. (1998) FASEB J. 12, 1507–1520
42. Pedesta, M., Zocchi, E., Pito, A., Usai, C., Franco, L., Bruzzone, S., Guida, L., Bacigalupo, A., Scadden, D. T., Walseh, T. F., De Flora, A., and Daga, A. (2000) FASEB J. 14, 680–690
43. Zocchi, E., Usai, C., Guida, L., Franco, L., Bruzzone, S., Passalacqua, M., and De Flora, A. (1999) FASEB J. 13, 273–283
44. Fogarty, K. E., Kidd, J. F., Tuft, D. A., and Thorn, P. (2000) J. Physiol. 526, 515–526
45. Looms, D. K., Tritosaris, K., Nauntofte, B., and Dissing, S. (2001) Biochem. J. 355, 87–95
46. Yu, J. Z., Zhang, D. X., Zou, A. P., Campbell, W. B., and Li, P. L. (2000) Am. J. Physiol. 279, H873–H881