Evidence for an Intracellular ADP-ribosyl Cyclase/NAD⁺-glycohydrolase in Brain from CD38-deficient Mice*

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Cyclic ADP-ribose, a metabolite of NAD⁺, is known to modulate intracellular calcium levels and signaling in various cell types, including neural cells. The enzymes responsible for producing cyclic ADP-ribose in the cytoplasm of mammalian cells remain unknown; however, two mammalian enzymes that are capable of producing cyclic ADP-ribose extracellularly have been identified, CD38 and CD157. The present study investigated whether an ADP-ribosyl cyclase/NAD⁺-glycohydrolase independent of CD38 is present in brain tissue. To address this question, NAD⁺ metabolizing activities were accurately examined in developing and adult Cd38⁻/⁻ mouse brain protein extracts and cells. Low ADP-ribosyl cyclase and NAD⁺-glycohydrolase activities (in the range of pmol of product formed/mg of protein/min) were detected in Cd38⁻/⁻ brain at all developmental stages studied. Both activities were found to be associated with cell membranes. The activities were significantly higher in Triton X-100-treated neural cells compared with intact cells, suggesting an intracellular location of the novel cyclase. The cyclase and glycohydrolase activities were optimal at pH 6.0 and were inhibited by z-mercaptoethanol, which is distinct from those of CD157. Both activities were enhanced by guanosine 5'-O-(3-thiotriphosphate), a result suggesting that the novel enzyme may be regulated by a G protein-dependent mechanism. Altogether our results indicate the presence of an intracellular membrane-bound ADP-ribosyl cyclase/NAD⁺-glycohydrolase distinct from CD38 and from CD157 in mouse brain. This novel enzyme, which is more active in the developing brain than in the adult tissue, may play an important role in cyclic ADP-ribosyl-mediated calcium signaling during brain development as well as in adult tissue.

Calcium is a universal signaling molecule that is involved in a wide variety of processes in almost all cell types from procaroytes to eucaryotes. The increase in cytosolic calcium levels necessary for signal transduction is usually achieved by entry of extracellular calcium and amplification of the process by calcium released from intracellular storage sites. The intracellular calcium mobilization occurs via two major families of channels in the endoplasmic reticulum, i.e. the inositol 1,4,5-trisphosphate and the ryanodine receptors.

The amplification of cytosolic calcium has been shown to be regulated by cyclic ADP-ribose (cADPR),¹ a metabolite of NAD⁺, that modulates calcium-induced calcium release. This regulation of calcium mobilization via cADPR has been conserved during evolution (1, 2). The ryanodine receptors, at least some isoforms, are generally considered to be responsive to cADPR (3). However, effects of cADPR on the inositol 1,4,5-trisphosphate-induced calcium release and the binding of inositol 1,4,5-trisphosphate to its receptors were also described recently (4, 5). The cascade of cellular events leading to the control of intracellular calcium channels by cADPR remains to be clarified. One of the most intriguing questions concerns the identity of the enzyme(s) that is responsible for producing cADPR in the cytosol of mammalian cells.

Cyclic ADPR is known to be synthesized from NAD⁺ by enzymes having ADP-ribosyl cyclase activity. The first cyclase discovered was isolated from the ovotestis of Aplysia californica (6). More recently, the Aplysia cyclase was also found in the neurons of this mollusk (7). In mammals, two proteins having around 90% sequence identity with the Aplysia cyclase and capable of ADP-ribosyl cyclase activity have been identified: CD38, which is a transmembrane glycoprotein expressed in many tissues, (8, 9) and CD157 (BST-1), which is a glycosylphosphatidylinositol-anchored surface molecule predominantly expressed in bone marrow stroma (10, 11). Whereas the Aplysia enzyme is predominantly a cyclase and is soluble, the two mammalian enzymes are membrane-associated showing, in vitro, principally NAD⁺-glycohydrolase activity and a minor cyclase activity. Nevertheless a unifying mechanism has been proposed recently for the invertebrate and mammalian enzymes (12, 13). Cyclic ADPR, which is abundant in the brain (14, 15), has been shown to be involved in many calcium-dependent neural processes including transmitter release (7, 16), synaptic trans-

¹ The abbreviations used are: cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; NGD⁺/H11001, nicotinamide guanine dinucleotide; ADPβly cyclase, ADP-ribosyl cyclase; NADase, NAD⁺-glycohydrolase; GTPγS, guanosine 5'-O-(2-thiodiphosphate); ATPγS, adenosine 5'-O-(2-thiodiphosphate); GDPβS, guanosine 5'-O-(2-thiodiphosphate); Sol extract, soluble extract obtained after high speed centrifugation; P10 extract, membrane extract obtained after low speed centrifugation; F540 extract, membrane extract obtained after high speed centrifugation; HPLC, high pressure liquid chromatography; E, embryonic day; PN, postnatal day; MES, 2-(N-morpholino)ethanesulfonic acid.
In the assay with phosphatidylinositol-specific phospholipase C, the P540 extract (1 mg/ml) was incubated for 30 min at 32 °C and pH 7.0 in the presence of 1 unit of enzyme. To test the effect of high salt concentrations, the P540 extract (1 mg/ml) was incubated for 30 min at 4 °C with stirring in a buffer containing 500 mM KCl, 150 mM Tris-HCl at pH 8.0. Samples were subsequently centrifuged for 30 min at 540,000 g. The NAD+ metabolizing activities were then measured (15-h incubation of extract at 1 mg/ml with 60 mM NAD+; 2 mM EDTA at 32 °C and pH 6.0) in the pellet and supernatant and compared with the activity observed in the non-treated P540 extract.

In the assays with nucleotides, the P540 extracts (1 mg/ml) were incubated for 1 h at pH 7.0 with 300 µM cGMP, GTP-S, GTP-γS, or ATP-γS or with 1 mM GTP or ATP before NAD+ was added. Then the activities of the NAD+ metabolizing activity were performed (5-h incubation at pH 7.0). In the experiment performed with cGMP, 300 µM 3-isobutyl-1-methylxanthine were added to inhibit cGMP degradation.

Suspended cerebral hemisphere cells (4 x 10^6 cells in 200 µl of reaction medium, corresponding to an average of 3.5 mg of protein/ml) were incubated with 200 µM [14C]NAD+ (4 µCi/mmol) for 4 h at 37 °C and pH 7.5 in a saline medium (see above) either in the absence or presence of 1% (v/v) Triton X-100.

Standards were prepared by mixing products (1:1:1) formed by reacting [14C]NAD+ with Aplysia ADP-ribosyl cyclase (60 µM NAD+; 0.4 µg/ml enzyme; 15-min incubation at 32 °C), with nucleotide pyrophosphatase (60 µM NAD+, 0.04 units/ml enzyme; 15-min incubation at 32 °C), and with NAD+–glycohydrolase (NADase) (60 µM NAD+, 0.04 units/ml enzyme; 15-min incubation at 32 °C). All reactions were stopped at given times by adding 10% (v/v) perchloric acid, incubating for 10 min at 6 °C, and then centrifuging the samples (16,000 g for 10 min). After neutralization of the supernatant with K2CO3 and a short centrifugation of the sample to eliminate the precipitates, the supernatant containing the reaction products was analyzed by HPLC.

Enzymatic Reactions with NGD—Assays of GDP-ribosyl cyclase activity were performed with P540 protein extracts (1 mg/ml) using 1 mM NGD+ in 2 mM EDTA, 20 mM MES-NaOH at pH 6.0 and 32 °C. Extracts from adult Cd38–/– brains were tested within 22 h of incubation time. Extracts from E15 Cd38–/– brains were tested within 16 h of incubation time. Controls performed under exactly the same conditions, but with boiled P540 extract, were performed.

To avoid any destruction of the fluorescent reaction product cyclic GDP-ribosyl by perchloric acid, the proteins in the assay were eliminated by centrifugation at 540,000 g and 0 °C for 30 min followed by filtration of the supernatant through a Microsorb 10K filter (Gelman Laboratory, Northborough, MA). The reaction products were then analyzed by HPLC. Standards were prepared by reacting NGD+ with Aplysia ADP-ribosyl cyclase (20 µM NGD+, 0.4 µg/ml enzyme, 20 mM Tris-HCl at pH 7.0 and 32 °C for a 5-min incubation).

Enzymatic Reactions with [14C]NAD+—The [14C]NAD+ was synthesized from [14C]NAD+ using Aplysia cyclase. Then P540 extracts (2 mg/ml) were tested for cADPR hydrolysis activity by a 23-h incubation with 60 µM [14C]cADPR (6 µCi/mmol) in 2 mM EDTA, 20 mM MES-NaOH at pH 6.0 and 32 °C. Control experiments were performed under the same conditions but in the absence of the P540 extract.

Analysis by HPLC—HPLC analysis was performed on a 300–
3.0-mm µBondapak C18 column (Waters) equipped with a guard column (LiChrosorb® 4-4 LiChrospher® 100 RP-18 5 µm, Merck EuroLab) as in Ref. 30. Aliquots of 50–100 µl were injected and eluted isocratically at room temperature with a medium containing 10 mM ammonium phosphate buffer at pH 5.5 and 1% (v/v) acetonitrile.

The detection of the products was performed using an on-line radioactivity monitor (LB 506D, Berthold) after mixing eluting products with a scintillation liquid (Quickzint flow 302, Zinsser Analytic) using a pump (LP 5035, Berthold). Reaction products of NAD+ (ADP, cADPR, and AMP) were identified on the chromatograms by separately injecting standards. Cyclic ADP-ribose was further identified by its chemical transformation to AMP catalyzed by nucleotide pyrophosphatase. The NAD+ products were always separated in the same order; however, elution times could vary (particularly for NAD+). This was due to differences in the room temperature in which the experiment was performed or the age of the C18 column. We recommend running the standards every time the experiments were performed. Chromatograms of the standards are shown in two critical figures (Figs. 1 and 6).
Fluorometric detection of cyclic GDP-ribose was performed with an on-line fluorescence monitor (SFM25, Kontron Instruments).

RESULTS

Previous results have shown that the cADPR content of brain homogenates prepared from Cd38−/− mice is only marginally reduced when compared with wild type brain homogenates, suggesting the existence of an ADP-ribosyl cyclase distinct from CD38 in the brain (15). Surprisingly, however, NAD−glycohydrolase (28) and ADP/GDP-ribosyl cyclase (26) activities were not detected in Cd38−/− brain protein extracts. To reconcile these disparate results, we have suggested that a CD38-independent ADP-ribosyl cyclase with basal activity too low to be measurable using the usual assay conditions, which could be activated by unknown cellular mechanisms, may be present in brain tissue (26).

In the present study, we have designed experimental conditions to determine very low levels of cADPR and ADPR production and have re-examined the presence of ADP-ribosyl cyclase (ADPRyl cyclase) and NADase activities in different brain extracts and in brain cells isolated from Cd38−/− mouse.

Evidence for cADPR and ADPR Synthesis in a Membrane Fraction from Cd38−/− Mouse Brain—To detect the presence of a putative enzyme distinct from CD38 and able to transform NAD+ into cADPR in murine brain tissues, we have used a sensitive assay with [14C]NAD as substrate, and reactions products were detected by HPLC coupled to an on-line radioactivity detector. Several fractions from brains of Cd38−/− adult mice were tested, including total protein extract, Sol extract, and two membrane fractions obtained at low (P10 extract) and high speed (P540 extract) centrifugation (see “Experimental Procedures”). All the enzymatic assays were performed in the presence of EDTA, which inhibits nucleotide pyrophosphatases that may hydrolyze NAD+ and the formed ADPR (31, 32). The extracts were tested for their ability to convert NAD+ into cADPR and ADPR after incubation at 32 °C with NAD+ for 10 min to 25 h. Different concentrations of NAD+ (6 μM, 60 μM, and 3 mM) and two pH conditions (pH 6.0 and pH 7.0) were examined. The stability of NAD+, cADPR, and ADPR was controlled within the time of measurement using standards: under our experimental conditions, the decrease in content of the standards was found to be less than 3% in 25 h (not shown). The highest cyclase and glycohydrolase activities were observed when extracts were incubated with 60 μM NAD+ at pH 6.0.

Fig. 1 shows representative HPLC radiochromatograms obtained after reacting 60 μM NAD+ for 15 h at pH 6.0 with P10 (Fig. 1B), P540 (Fig. 1C), or Sol (Fig. 1D) extracts. Reactions with the P10 (Fig. 1B) and Sol (Fig. 1D) extracts yielded small peaks corresponding to ADPR and representing a conversion of 5.6 and 2.7% of NAD+, respectively. While the amount of ADPR present in the Sol extract was equivalent to the contaminating tissue of adult Cd38−/− mouse brain extracts (not shown), the amount of ADPR present in the P10 membrane extract was greater and suggested that the P10 fraction had very low NADase activity. No peak corresponding to cADPR was detected with either of these extracts. In contrast, incubation of NAD+ with the P540 membranes yielded ADPR and cADPR peaks (Fig. 1C and Fig. 2A, which corresponds to a 3-fold magnification of the chromatogram shown in Fig. 1C). Under our experimental conditions, these two reaction products represented a conversion of respectively 15.4 and 1.4% of the reactive NAD+. Heat inactivation of the P540 fraction (10 min at 100 °C) before incubation with [14C]NAD+ completely abolished the formation of both products demonstrating their enzymatic origin (not shown). The identity of the ADPR peak was confirmed by treatment with nucleotide pyrophosphatase, which yielded AMP. Similarly the identity of the cADPR peak was assessed by an absence of reaction with nucleotide pyrophosphatase and its hydrolytic transformation into ADPR by heat treatment (15 min at 100 °C). Cyclic ADP-ribose and

![Fig. 1. Analysis of NAD+ metabolizing activities of adult Cd38−/− mouse brain extracts.](image)

![Fig. 2. ADPR and cADPR formation by a membrane extract from brains of adult Cd38−/− mice.](image)
ADPR formation increased linearly with the protein concentration of the P540 extract as shown in Fig. 2B.

Together these results demonstrate the presence of low basal ADPRyl cyclase and NADase activities, evocative of a multifunctional enzyme, in a protein fraction isolated from Cd38−/− mouse brain. This P540 fraction, obtained after high speed centrifugation, was shown previously to be enriched in endoplasmic reticulum and plasma membranes (26).

**Rates of Activities in the Membrane Fraction Isolated from Adult Cd38−/− Mouse Brain**—Fig. 3 shows the time course of cADPR and ADPR production by the P540 membrane fraction at pH 6.0 (Fig. 3A) or 7.0 (Fig. 3C). At pH 6.0, both cADPR and ADPR increased linearly during the 25 h of measurement, reaching average values of 1.14 and 10.5 nmol, respectively. The deduced rates of reaction are 0.8 pmol of cADPR and 7 pmol of ADPR formed/mg of extract/min. Thus, at this pH, the ratio between ADPRyl cyclase and NADase activities is ~1:100. Under these same experimental conditions, the NADase activity observed in a P540 brain extract from normal mice was approximately 3 orders of magnitude higher (4.6 nmol of product formed/mg/min, not shown). Thus, under basal in vitro conditions, CD38 accounts for the vast majority of NADase activity. Interestingly minimal cADPR was formed when incubating the P540 extracts from normal mouse brains with NAD+, correlating with the ratio of 1:100 between cyclase and glycohydrolase activities of CD38 already estimated (33).

The enzyme reactions shown in Fig. 3, A and C, included 2 mM EDTA in the reaction buffer to inhibit the pyrophosphatase activity present in the extracts. To exclude the possibility that the EDTA was also inhibiting the activities of the novel brain enzyme by chelating necessary cations, we measured ADP-ribosyl cyclase and NADase activity at pH 6.0 (Fig. 3B) and 7.0 (Fig. 3D) in the P540 extracts in the presence and absence of 2 mM EDTA. As expected, the presence of EDTA resulted in increased accumulation of ADPR, most likely by inhibiting the endogenous nucleotide pyrophosphatase activities (31, 32). Importantly, however, the presence of EDTA had absolutely no effect on cADPR production, indicating that the cyclase activity of the novel brain enzyme is not dependent on the presence of divalent cations.

Together these results indicate that the brain contains a novel non-CD38 enzyme that can catalyze the formation of both ADPR and cADPR from NAD⁺. This enzyme has optimal ADPRyl cyclase and NADase activities at pH 6.0 compared with pH 7.0 but the ratio between the activities (1:8–9) is quite similar under both pH conditions. The data also indicate that the NADase activity observed in the Cd38−/− brain P540 membrane extract has a rate about 1000-fold lower than the rate obtained with the same extract isolated from adult brain of a wild type mouse. Although the basal activity of the novel enzyme is lower than that of CD38, this enzyme is a more efficient cyclase than CD38 in that cADPR accounts for up to 10% of the products formed. Finally the novel brain enzyme appears to be very stable at 32 °C during the time of measurement.

Characterization of the Novel Multifunctional Enzyme Present in Cd38−/− Adult Brain—We wanted to better characterize the new cyclase, which is independent of CD38 but which shares with CD38 similar multifunctional features, present in the brain tissue. At first, we compared it with CD157, which is the only other mammalian enzyme described so far that is endowed with similar catalytic properties (10, 34, 35).

Fig. 4 shows that both ADPRyl cyclase (in Fig. 4A) and NADase (in Fig. 4B) activities present in the Cd38−/− P540 extract are optimal at pH 6.0 in the presence of EDTA. Both enzyme activities decreased at higher or lower pH conditions. These results are clearly different from the results obtained by Hirata et al. (10) showing that, in the presence of EDTA, both ADPRyl cyclase and NADase activities of a soluble form of human CD157 increased as the reactions were carried out under more acidic pH conditions. Moreover the ratio between ADPRyl cyclase and NADase activities of recombinant CD157 was 2:1 at pH 6.0 and is therefore far from the ratio we observed in the present study for the novel non-CD38 enzyme (1:9 at pH 6.0).

We next studied the effect of zinc ions on the ADPRyl cyclase activity present in the P540 extract from Cd38−/− mice since zinc is known to strongly activate both murine (34) and human (10) CD157 cyclase activities. In sharp contrast to such results, when this activity was examined at pH 7.0 in the presence of 1 mM ZnCl₂ (Fig. 4C), we observed a drastic inhibition of cADPR formation: the average production was decreased more than 5-fold (from 0.56 to 0.1 nmol of cADPR formed in 15 h/mg of protein).

We have also tested the effect of treating the P540 extract with phosphatidylinositol-specific phospholipase C, which has
been shown to release from membranes and solubilize glycosylphosphatidylinositol-anchored CD157 (27). No ADP-ribosyl cyclase and NADase activities were solubilized by this phospholipase (see conditions under "Experimental Procedures"). No activities could be detected in the supernatant, whereas both ADP-ribosyl cyclase and NADase activities were recovered (about 80%) associated with the membranes. This suggests that the novel cyclase/NADase from mouse brain is not glycosylphosphatidylinositol-anchored to the membrane.

We have tested the effect of high salt concentrations to determine whether the interaction of the novel enzyme with the membranes could be of electrophoretic origin. The P540 fraction was therefore incubated with 500 mM KCl at pH 8.0; however, no enzyme activity could be recovered in the supernatant after centrifugation (see conditions under "Experimental Procedures").

In a previous work (26), we have tested the capacity of crude protein fractions from Cd38<sup>−/−</sup> brain to utilize NGD<sup>+</sup> as a substrate. Kinetic fluorometric measurements were performed, and no GDP-ribosyl cyclase activity could be detected. We re-examined the possible formation of cyclic GDP-ribose by the P540 fraction under experimental conditions that allowed us to detect the transformation of [14C]cADPR from [14C]NAD<sup>+</sup>, i.e. long incubation times and high protein concentrations. Moreover the putative formation of cyclic GDP-ribose was now analyzed, after elimination of the proteins from the assay medium, with an HPLC system connected to an on-line fluorescence detector. This assay, which proved to be much more sensitive than our previous one (26) (i.e. about 0.1 nmol of cyclic GDP-ribose corresponding to 0.1% of NGD<sup>+</sup> transformation should have been detectable), did not allow us to observe any GDP-ribosyl cyclase activity in the P540 fraction (not shown).

Finally we tested the ability of the P540 extract to hydrolyze cADPR. This is an important point because the low yield of transformation of NAD<sup>+</sup> into cADPR, compared with ADPR, could <i>a priori</i> also originate from a potent cyclic ADP-ribose hydrolase activity. Experiments were conducted under conditions that allowed for easy detection of the NAD<sup>+</sup>-glycohydrodase activity (i.e. long incubation time, 2 mg/ml protein extract, and in the presence of 60 μM [14C]cADPR; see "Experimental Procedures"). Under these conditions no cADPR hydrolysis was observed, although a transformation of 3% of the labeled cADPR into ADPR was detectable by the highly sensitive HPLC method (not shown).

Altogether these results argue that the ADP-ribosyl cyclase/NAD<sup>+</sup>-glycohydrodase activities present in Cd38<sup>−/−</sup> brain are different from those of CD157 and should rather be attributed to a new multifunctional enzyme. This membrane-bound enzyme, unlike CD38 and CD157, functions best at pH 6.0, is inhibited by zinc ions, and seems unable to catalyze the cyclization of NGD<sup>+</sup>. Although under the present experimental conditions we could not measure any transformation, this new enzyme is also most probably a poor cADPR hydrolase as are CD38 and the other Cd38-like enzymes.

**Measurements of Activities during Cd38<sup>−/−</sup> Mouse Brain Development**—We wished to analyze the presence of both cyclase and hydrolase activities in the developing Cd38<sup>−/−</sup> brain tissue. Soluble and membrane fractions were prepared from embryonic (E15) and neonatal (PN1) Cd38<sup>−/−</sup> mouse brain and were tested for their ability to synthesize cADPR and ADPR under the same experimental conditions as those used to analyze the adult extract. As previously shown with extracts obtained from adult brain tissue, no NADase or ADP-ribosyl cyclase activity was observed in the Sol extract, and only minimal NADase activity was observed in the P10 membrane extract (not shown). However, as shown in Fig. 5, the HPLC profiles obtained with the P540 extract isolated from E15 (in Fig. 5A) and PN1 (in Fig. 5C) brains after 15 h of incubation with radiolabeled NAD<sup>+</sup> indicated the presence of both cyclase and glycohydrodase activities. When compared with the profile obtained in the same condition with the adult P540 extract (in Fig. 5D), same as in Fig. 1C), one can observe that the highest activities were present in the developing tissues. Indeed 3.4 and 4.1% of the reactive NAD<sup>+</sup> were converted to cADPR in the reaction containing the E15 and PN1 extracts, respectively, while only 1.4% of the NAD<sup>+</sup> was converted to cADPR in the reaction containing the adult extract. Likewise 40.8 and 50.3% of the reactive NAD<sup>+</sup> was converted to ADPR in the reaction containing the E15 and PN1 extracts, respectively, whereas only 15.4% of the NAD<sup>+</sup> was converted to ADPR in the reaction containing the adult extract. The time course of product formation by the E15 extract is shown in Fig. 5B. ADPR and cADPR increased linearly over time reaching average values of 1.8 and
22.8 nmol formed in 15 h, respectively. The deduced rates of reaction for the E15 P540 extracts are 2 pmol of cADPR and 25 pmol of ADPR formed/mg/min with an ADPRyl cyclase and NADase activity ratio of about 1:12. Thus, although the reaction rates of the novel enzyme are about 3-fold higher in embryonic brain tissue compared with adult brain tissue, the ratio between the cyclase and NADase activities are on the order of 1:10 for both extracts.

We have also tested the transformation of NGD\(^+\) by the enzyme present in the P540 fraction of embryonic brain under experimental conditions that allowed the detection of the transformation of NAD\(^+\) into cADPR (see “Experimental Procedures”). As in the adult tissue, no GDP-ribosyl cyclase activity could be detected (not shown).

Altogether these results demonstrate that a novel enzyme with low basal ADPRyl cyclase and NADase activities is present in the brain of developing and adult mice that are devoid of CD38. Furthermore they show that these activities are about 3-fold higher in embryonic brain tissue compared with adult brain tissue, suggesting that this enzyme is either more highly expressed or more active in developing brain tissue.

**NAD\(^+\) Metabolizing Activities of Intact and Permeabilized CD38-deficient Neural Cells**—We next wished to determine whether the novel enzyme revealed in the CD38\(^{-/-}\) brain has ectocellular or intracellular activity. We therefore tested the NAD\(^+\) metabolizing activities of intact and permeabilized cells isolated from neonate cerebral hemispheres. Freshly dissociated cells (4 \(\times\) 10\(^6\)/assay) were tested for their ability to transform \[^{14}\text{C}]\text{NAD}^+ (200 \mu\text{M}) into cADPR and ADPR after incubating for 4 h at 37 °C and pH 7.5 in the presence of 5 mM EDTA. Fig. 6 shows representative \[^{14}\text{C}\]HPLC profiles obtained after reacting the labeled substrate with intact (Fig. 6B) and Triton X-100-treated (Fig. 6C) cells from the same preparation. In the reaction with intact cells (Fig. 6B), a peak corresponding to ADPR was observed (7.2% of NAD\(^+\)), whereas no cADPR peak could be detected. In the reaction with Triton X-100-treated cells (Fig. 6C), about 52% of the reactive NAD\(^+\) was converted to ADPR, and 4.4% was converted to the cyclic compound. These experiments were repeated with three different sets of cells giving the average results presented in Fig. 6D. In the reaction with intact cells, an average of 0.7 nmol of ADPR/mg of protein were formed within 4 h, corresponding to a rate of 3 pmol of ADPR produced/mg/min. Under the same experimental conditions, the presence of Triton X-100 resulted in an 11-fold increase in the production of ADPR, leading to an average ADPR production rate of 34 pmol/mg/min. Moreover the Triton X-100-treated cells produced 0.8 nmol of cADPR/mg of protein in 4 h, corresponding to a rate of 3.2 pmol of cADPR produced/mg/min. The ratio between ADP-ribosyl cyclase and NADase activities measured with the permeabilized cells was therefore 1:10, a rate that is essentially identical to the one measured with the P540 membrane extract from whole brain. It is also interesting to note that the cyclase and NADase activity rates obtained with the cerebral hemisphere cells were in the same range as the rates measured with the P540 extracts (pmol/mg/min).

The measurement of much larger ADPRyl cyclase and NADase activities in permeabilized cells than in intact cells strongly suggests that these activities are due to an enzyme that is localized to intracellular membranes or to the plasma membrane with its catalytic site located in the cytosol. The low NADase activity observed in the intact cells could be due to the presence of dead and therefore leaky cells from the suspension (which we estimated represented around 10% of the total preparation, see “Experimental Procedures”).

**Effect of Guanine Nucleotides on ADPRyl Cyclase and NADase Activities from Adult CD38\(^{-/-}\) Mouse Brain Extract**—We have established the existence of a novel ADPRyl cyclase/NADase enzyme expressed in the brain of developing and adult mice. However, since this enzyme has low basal activities, we have looked for ways to enhance its activity. Because cGMP and G proteins were already proposed to activate ADPRyl cyclase activity in sea urchin oocytes and in some mammalian cells including neural cells (19, 20, 36–38), we decided to test the effect of these nucleotides on the NAD\(^+\) metabolizing activities of the CD38\(^{-/-}\) P540 brain extract.

The adult P540 extract was preincubated for 1 h with 300 \(\mu\text{M}\) cGMP, GTP\(\gamma\text{S},\) GTP\(\beta\text{S,}\) or ATP\(\gamma\text{S}\) or with 1 mM GTP. \[^{14}\text{C}]\text{NAD}^+ was then added, and the extracts were incubated at pH 7.0 and 32 °C for an additional 3 h. Both incubations (with the different nucleotides and then with the substrate) were performed in the absence of EDTA. Fig. 7 shows the average results of at least three different experiments.

As expected after this short incubation time, no cADPR and only a small quantity of ADPR (0.26 nmol/mg) could be detected in the control experiments that were performed in the absence of added nucleotides. The presence of cGMP did not produce...
any detectable effect on either ADPR or cADPR formation. However, the presence of GTPγS induced the production of both cADPR (0.33 nmol/mg) and ADPR (which increases to 3.4 nmol/mg, corresponding to a 13-fold increase in activity). In the presence of GTP, the production of ADPR was also significantly increased (to 2.6 nmol/mg, corresponding to a 10-fold rise). However, no cADPR production could be detected. GTP appears thus less effective than its non-hydrolyzable analogue. The rate of ADPRylation cyclase reaction deduced from the experiments performed in the presence of GTPγS was ~2 pmol of cADPR formed/min. Thus, compared with the experiments performed under similar experimental conditions in the absence of GTPγS (Fig. 3D), the cADPR synthesis rate increased about 4-fold when adding GTPγS.

The ADPR production was increased, but only slightly, in the presence of GDPγS (to 0.62 nmol/mg) or ATPγS (to 0.55 nmol/mg). No cADPR production could be detected in the presence of either of these nucleotides. Furthermore the presence of 1 mM ATP did not modify the result obtained with a 3-h incubation time in the absence of nucleotide (not shown). All these results show that the effect of GTPγS on the ADPRylation cyclase and NADase activities appears clearly dependent on the presence of the guanosine and triphosphate moieties of this nucleotide. Moreover no effect of GTPγS could be observed in the presence of 2 mM EDTA (not shown), showing that the stimulation of activities by this nucleotide requires divalent ions.

Our results therefore show that both cADPR and ADPR synthesis activities of the novel enzyme are significantly stimulated by the presence of GTPγS. Thiophosphorylations are most probably not involved in this activation since ATPγS has only a very limited effect on the NADase activity and no effect on the cyclase activity compared with GTPγS.

DISCUSSION

In the present work, we clearly reveal the existence of a novel NAD⁺-metabolizing enzyme in brain tissue. Interestingly the novel enzyme appears to have both ADP-ribose cyclase and NAD⁺-glycohydrolase activities just like CD38 and CD157. When protein extracts from the Cd38⁻/⁻ brain were fractionated (Fig. 1), these activities were found primarily in a membrane fraction that we have shown previously to be enriched in endoplasmic reticulum and plasma membranes (26). When studied in CD38-deficient neural brain cells (Fig. 6), the cyclase and NADase activities were considerably higher in Triton X-100-treated cells than in intact cells. Our results therefore strongly suggest that the novel enzyme is membrane-associated and is intracellulatively located. The CD38-independent ADP-ribosyl cyclase and NAD⁺-glycohydrolase activities were shown to be present in the developing brain tissue, studied at embryonic day 15 and postnatal day 1. In contrast to CD38-dependent NAD⁺ metabolizing activities (26), the enzyme activities of the novel enzyme were higher in the developing brain tissue compared with the adult tissue.

We compared the biochemical characteristics of the enzyme found in the Cd38⁻/⁻ brain to that of the only other mammalian ADP-ribosyl cyclase/NAD⁺-glycohydrolase known, the glycosylphosphatidylinositol-anchored surface molecule CD157 (10, 11). Such comparison was not easy since CD157 has not been as extensively studied as CD38. Altogether our data argue that the ADP-ribose cyclase and NAD⁺-glycohydrolase activities measured in the Cd38⁻/⁻ mouse brain tissue are unlikely to be due to CD157. Importantly our data show that this enzyme is intracellular (either plasma membrane-associated or linked to an organelle-associated membrane), is most active at pH 6.0, and is inhibited by zinc ions (Fig. 4). This is in contradiction to CD157, which is expressed on the plasma membrane with its catalytic site outside the cell and is activated at more acidic pH conditions in the presence of zinc ions (10, 34, 35).

Thus, our results are consistent with studies showing the absence of detectable levels of CD157 transcripts in brain tissue by Northern blots (11, 27) and strongly suggest that the brain-associated cyclase described in this study is novel. In agreement with this conclusion, we found that the novel enzyme is unable to catalyze the cyclization reaction with the NAD⁺ analogue NGD⁺, while CD38, CD157, and the Aplysia cyclase can all utilize NGD⁺ as a substrate. It is possible, however, that the novel cyclase described here is present in tissues in addition to the brain. Indeed an ADP-ribosyl cyclase unable to utilize NGD⁺ as a substrate was identified in lymphocyte and implicated in the signaling cascade following activation of T-cell receptors (39).

The NADase activity from adult Cd38⁻/⁻ brain examined in vitro using protein extracts and cells was 3 orders of magnitude lower compared with the CD38-dependent NADase activities found in the same preparations isolated from normal mouse brains; i.e., it was in the range of pmol of products formed/mg of proteins/min (see Fig. 3) instead of nmol/mg/min (26). However,
it is noteworthy that the ADP-ribosyl cyclase activity is most easily revealed in brain extracts or cells isolated from Cd38−/− mice than in the same preparations obtained from wild type mice. In the latter preparations, the CD38-dependent NADase activity predominates so much that the formation of the cyclic metabolite is difficult to detect (26). Relative to CD38, the novel brain ADP-ribosyl cyclase/NAD−-glycohydrolase favors the formation of the cyclic compound. Using the data generated in this study, we can estimate the approximate time needed for the novel enzyme to renew the homeostatic levels of cADPR in the brain. In permeabilized cells from Cd38−/− neonate mouse cerebral hemispheres, the rate of ADP-ribosyl cyclase activity was found to be 3.2 pmol/mg/min under our experimental conditions. If we assume that the cyclase activity is approximately the same order of magnitude in adult brain cells, and knowing that the cADPR content was found to be 3 pmol/mg of protein in adult Cd38−/− mouse brain tissue (15), that means that the cADPR levels in the brain may be renewed within 1 min or a few minutes by the novel non-CD38 enzyme.

In addition, the low basal activities of the enzyme measured in brain protein extracts or cells isolated from Cd38−/− mice may not represent what would be observed under normal physiologic parameters, particularly if the enzyme activity is stimulated by specific cellular conditions. This could be the case for the novel enzyme since we found that both ADP-ribosyl cyclase and NADase activities from Cd38−/− mouse brain tissue were enhanced by the presence of GTP-γS (Fig. 7). These latter results suggest that the activity of the novel enzyme present in the brain tissue could be up-regulated either directly by GTP or via a G protein-mediated pathway. Coupling between G proteins and unidentified intracellular ADP-ribosyl cyclases has already been described in mammalian cells and particularly in neuronal cells. The authors have described regulation of an ADP-ribosyl cyclase after stimulation of receptors, such as muscarinic acetylcholine receptors (37), angiotensin II and adrenergic receptors (40, 41), and serotonin 5A receptors (42). The involvement of a G protein-regulated ADP-ribosyl cyclase in Ca2+ mobilization induced by activation of metabotropic glutamate receptors in dopamine neurons was also depicted recently (20). Our present work may indicate that the novel ADP-ribosyl cyclase, independent of CD38, is a candidate to be involved in these newly described signaling pathways. Experiments will be conducted in the future using Cd38−/− brain cells to uncover the signaling cascades involving this novel cyclase and possibly a G protein.

Our data demonstrating the existence of a novel ADP-ribosyl cyclase/NAD−-glycohydrolase in brain tissue may explain some of our earlier results showing that cADPR levels are only slightly reduced in extracts prepared from brains isolated from the Cd38 knock-out mice compared with extracts prepared from normal mice (15). Altogether our results show that the novel non-CD38 enzyme studied in the present work appears to play an important role in regulating cADPR levels in the brain.

For the last several years, with regard to the biosynthesis of intracellular cADPR, the question has been raised whether an intracellular soluble enzyme with predominant cyclase activity, similar to the Aplysia ADP-ribosyl cyclase/NAD−-glycohydrolase (12, 43), is present in mammalian cells. Our present results, along with our previous studies (26), show that, at least in brain, no enzyme with these properties could be found. Rather our data point to the presence in the brain of two distinct membrane-bound enzymes exhibiting in vitro ADP-ribosyl cyclase activities: (1) CD38, which is localized in plasma and intracellular membranes and whose expression increases during brain development, and (2) an unidentified novel intracellular enzyme that may be regulated by a G protein and whose activity is higher in the developing brain compared with the adult brain. The latter enzyme appears to be a good candidate to be involved in the production of the intracellular calcium signaling molecule cADPR in brain and useful for modulating neurotransmitter release, cell excitability, and synaptic plasticity.

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