

# CD38 is the major enzyme responsible for synthesis of nicotinic acid–adenine dinucleotide phosphate in mammalian tissues

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In the present study, we have determined the role of the enzyme CD38 upon the synthesis of the Ca<sup>2+</sup>-releasing nucleotide nicotinic acid–adenine dinucleotide phosphate (NAADP). In rat tissues, we observed that the capacity for NAADP synthesis could be co-immunoprecipitated with CD38 using an anti-CD38 antibody. Furthermore, we observed that several tissues from CD38 knockout mice had no capacity for the synthesis of this nucleotide. In addition, CD38 was also identified as the major

enzyme responsible for the synthesis of the second messenger cyclic ADP-ribose. These observations lead to the conclusion that CD38 is the major enzyme responsible for the synthesis of NAADP and cyclic ADP-ribose, and raises the possibility of a new signalling pathway where two different Ca<sup>2+</sup>-releasing nucleotides are synthesized by the same enzyme.

**Key words:** Ca<sup>2+</sup> release, cyclic ADP-ribose, knockout mice.

## INTRODUCTION

Release of Ca<sup>2+</sup> from intracellular stores is a widespread component of several signalling pathways [1,2]. Nicotinic acid–adenine dinucleotide phosphate (NAADP) is a recently discovered nucleotide with intracellular Ca<sup>2+</sup>-releasing properties [3–9]. NAADP-induced Ca<sup>2+</sup> release was first described in sea urchin egg homogenates [5]. The Ca<sup>2+</sup>-release mechanism elicited by NAADP differs in many ways from the Ca<sup>2+</sup> release controlled by cyclic ADP-ribose (cADPR) and inositol 1,4,5-trisphosphate (IP<sub>3</sub>) [3–13]. Properties of this Ca<sup>2+</sup>-releasing molecule include: (1) the absence of regulation by intracellular bivalent cations Mg<sup>2+</sup> and Ca<sup>2+</sup> [7,8]; (2) NAADP-induced Ca<sup>2+</sup> release is fully inactivated by exposure to low concentrations of NAADP [13]; and (3) the Ca<sup>2+</sup> release induced by NAADP appears to be insensitive to a wide range of changes in pH [9]. These characteristics make NAADP a unique trigger of intracellular Ca<sup>2+</sup> [3–13]. Recently, NAADP-induced Ca<sup>2+</sup> release has also been extended to several mammalian cells and tissues [14–19]. In fact, in pancreatic cells NAADP-regulated Ca<sup>2+</sup> stores appear to be responsible for the initiation of the Ca<sup>2+</sup> spiking induced by cholecystokinin [14,15].

In view of the potential role of NAADP as a regulator of intracellular Ca<sup>2+</sup>, we have explored the mechanisms of NAADP synthesis in mammalian tissues. Synthesis of NAADP by a base-exchange reaction has been described in several mammalian tissues, including brain, heart, liver, spleen and kidney [19,20]. Furthermore, it has also been reported that the so-called ADP-ribosyl cyclase is capable of catalysing the synthesis of NAADP [21,22]. This enzyme was first described as being responsible for the synthesis of yet another intracellular Ca<sup>2+</sup> regulator, cADPR [23]. In mammalian cells the ADP-ribosyl cyclase, named CD38, is the major enzyme involved in the synthesis of cADPR [24–29]. Furthermore, in HL-60 cells, CD38 has also been reported to catalyse the synthesis of NAADP [21,22]. However, it is not known whether CD38 is the major enzyme involved in the synthesis of NAADP in mammalian tissues.

In the present study, we provide experimental evidence to suggest that CD38 is the major enzyme responsible for NAADP synthesis in mammalian tissues. These data raise the possibility of a converging signalling pathway where two distinct intracellular Ca<sup>2+</sup> signalling molecules, namely NAADP and cADPR, could be synthesized by the same enzyme.

## MATERIALS AND METHODS

### Knockout mice

CD38 knockout [CD38(–/–)] mice were generated by homologous recombination. The generation and genotyping have been described previously [26,30]. The mice used for each experiment were from the same litter or family.

### Preparation of crude cytosolic extract

Tissues were harvested from adult male Sprague–Dawley rats (200–250 g body weight) and mice. Animals were killed by pentobarbital anaesthesia. Brain, spleen, liver, heart, lung, skeletal muscle, pancreas and kidney cortex were quickly dissected, chilled, and minced in an ice-cold solution of 20 mM Tris/HCl (pH 7.2), 0.25 M sucrose and 20 mg/ml leupeptin. Tissues (20%, w/v) were homogenized in a Dounce homogenizer using 4–5 strokes and centrifuged at 2000 g for 10 min at 4 °C. The supernatants were collected and used for the determination of NAADP and cADPR synthesis. Only freshly prepared tissue homogenates were used in this study.

### Subcellular fractionation

Plasma membrane fractions were prepared using centrifugation in a discontinuous sucrose gradient as described previously [31]. The final pellet was washed and resuspended in 40 mM Tris/HCl (pH 7.2) containing 0.25 M sucrose.

Abbreviations used: cADPR, cyclic ADP-ribose; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid–adenine dinucleotide phosphate.

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Mitochondrial fractions were prepared as described previously [31]. Briefly, tissues were minced and a 30% (w/v) solution was prepared in a homogenizing buffer [2 mM Hepes/KOH (pH 7.4), 0.07 M sucrose, 0.21 M D-mannitol and 0.05% BSA]. The tissues were homogenized with four strokes in a glass/Teflon homogenizer at 1500 rev./min. The homogenates were diluted to 10% (w/v) in homogenizing buffer, filtered through four layers of gauze, and centrifuged at 660 g for 15 min. The resulting supernatant was centrifuged at 6800 g for 15 min and the pellet obtained was resuspended in homogenizing buffer and centrifuged at 9770 g for 15 min. This washing procedure was repeated twice, and the washed pellet was resuspended in 50 mM Tris/HCl (pH 7.2) containing 0.25 M sucrose. This final pellet was termed the mitochondrial fraction. No activity of the sarcoplasmic reticulum enzymes thapsigargin-sensitive Ca<sup>2+</sup> ATPase and glucose-6-phosphatase was detected in this fraction or in the plasma membrane fraction.

Microsomal and cytosolic fractions were prepared using the supernatant generated after the centrifugation at 6890 g for 15 min, as described above for the mitochondrial preparation. The supernatant was further centrifuged at 20000 g for 20 min, and the resulting supernatant was collected and centrifuged at 100000 g for 60 min in an ultracentrifuge. The supernatant was denoted as the cytosol and the pellet, after being resuspended in 40 mM Tris/HCl (pH 7.2) containing 0.25 M sucrose, was termed the microsomal fraction.

Nuclear fractions were prepared as described previously [32]. Briefly, tissue was homogenized in 10 mM potassium phosphate buffer (pH 7.2) containing 1.3 M sucrose and 1 mM MgCl<sub>2</sub>. The homogenate was centrifuged at 1000 g for 15 min at 4 °C. The pellet was suspended in 10 mM potassium phosphate buffer (pH 7.2) containing 2.2 M sucrose and centrifuged at 100000 g for 1 h. The final nuclear pellet was suspended in 20 mM Tris/HCl (pH 7.2) containing 0.25 M sucrose and centrifuged for 1000 g for 10 min at 4 °C. The resulting pellet was resuspended in 20 mM Tris/HCl (pH 7.2) containing 0.25 M sucrose.

### NAADP synthesis by a base-exchange reaction

Membrane fractions (1 mg/ml) were incubated with 1 mM NADP and 40 mM nicotinic acid at 37 °C in a buffer containing 40 mM triethanolamine-acetic acid buffer (pH 7.2). Aliquots (3–7 µl) were removed after different incubation times and NAADP content was determined using a combination of the sea urchin egg homogenate bioassay and HPLC analysis of nucleotides.

### cADPR synthesis

ADP-ribosyl cyclase activity was assayed using NAD<sup>+</sup> as described previously [31]. The enzyme preparations were incubated at 1 mg/ml in a buffer containing 40 mM Tris/HCl (pH 7.2), 1 mM NAD<sup>+</sup> and 0.25 M sucrose at 37 °C. Aliquots (3–7 µl), prior to and after incubation, were assayed for cADPR content using the sea urchin egg homogenate bioassay and HPLC as described previously [31]. Specific activity was expressed as nmol of cADPR produced/min per mg of protein.

ADP-ribosyl cyclase activity was also determined using the nicotinamide-guanine dinucleotide technique as described previously [31]. Enzyme preparations were incubated in 40 mM Tris/HCl (pH 7.2) containing 0.2 mM nicotinamide guanine dinucleotide and 0.25 M sucrose at 37 °C. Activity was determined using a fluorimetric assay at 300 nm excitation and 410 nm emission [31].

### <sup>45</sup>Ca<sup>2+</sup>-releasing assay from microsomes

NAADP-induced Ca<sup>2+</sup> release was performed as described previously [18]. Briefly, microsomes (100 µg) from CD 38(+ / +) wild-type mice were passively loaded by incubation for 3 h at 17 °C in a buffer containing 25 mM Hepes (pH 7.2), 100 mM KCl, 1 mM CaCl<sub>2</sub> and 1 µCi of <sup>45</sup>Ca<sup>2+</sup>. The release of Ca<sup>2+</sup> from loaded microsomes was initiated by a 10-fold dilution of the microsomes in a buffer containing 25 mM Hepes (pH 7.2), 100 mM KCl, 1 mM EGTA and 1 mM MgCl<sub>2</sub>. After incubation for 10 s, the suspension was further diluted in buffer [25 mM Hepes (pH 7.2), 100 mM KCl and 1 mM CaCl<sub>2</sub>] containing 10 µM NAADP. <sup>45</sup>Ca<sup>2+</sup> release was stopped after 60 and 120 s by a rapid filtration technique using a Whatman GF/B filter. The filters were rinsed with 25 mM Hepes (pH 7.2) containing 100 mM KCl, 1 mM EGTA and 1 mM MgCl<sub>2</sub>, and the radioactivity remaining on the filter was determined by liquid-scintillation counting.

### Synthesis of nucleotide standards

cADPR was synthesized by *Aplysia* ADP-ribosyl cyclase, using homogenized *Aplysia* ovotestes as described previously [6]. NAADP was synthesized via the base-exchange reaction catalysed by NAD<sup>+</sup> glycohydrolase, with minor modification [6]. Nicotinic acid (100 mM) and β-NADP<sup>+</sup> (10 mM) were incubated in 20 mM triethanolamine (pH 7.6) with 0.2 g/ml porcine brain NAD(P)ase (Sigma) for 3 h at 37 °C. The reaction was stopped by acetone precipitation.

After acetone precipitation, the nucleotides were purified by HPLC anion-exchange chromatography using an AG MP-1 resin (Bio-Rad, Hercules, CA, U.S.A.) packed into a 1 cm × 10 cm column. The nucleotides were eluted with a non-linear gradient of 150 mM trifluoroacetic acid and water, and monitored by UV absorption at 250 nm. Purified cADPR or NAADP were evaporated to dryness in a SpeedVac concentrator. cADPR and NAADP used in all experiments were at least 97% pure as determined by HPLC.

### Detection of nucleotides

#### Sea urchin egg homogenate bioassay

Homogenates from *Lytechinus pictus* eggs were prepared as described previously, with minor modification [6]. Briefly, eggs were obtained by injection of 0.5 M KCl into the coelomic cavity, and collected in artificial sea water. The jelly coats were washed from the eggs by several passages through 80-mm-mesh silk. The eggs were then washed once in artificial sea water, twice in Ca<sup>2+</sup>-free sea water containing 1 mM EGTA, twice in Ca<sup>2+</sup>-free water without EGTA and once in buffer containing 20 mM Hepes buffer (pH 7.2), 250 mM N-methylglucamine, 250 mM potassium gluconate, 1 mM MgCl<sub>2</sub>, 2 units/ml creatine kinase, 4 mM phosphocreatine, 1 mM ATP, 25 mg/ml leupeptin, 20 mg/ml aprotinin and 100 mg/ml soybean trypsin inhibitor. A suspension (25%, w/v) was prepared by homogenization with 4–5 strokes in a Dounce homogenizer with a type A pestle. The homogenate was then centrifuged for 10–12 s at 13000 g at 4 °C and the supernatant was collected and stored in 1 ml aliquots at –70 °C. Frozen homogenates were thawed in a 17 °C water bath and diluted to 1.25% (v/v) with an intracellular media solution containing 20 mM Hepes (pH 7.2), 250 mM N-methylglucamine, 250 mM potassium gluconate, 1 mM MgCl<sub>2</sub>, 2 units/ml creatine kinase, 4 mM phosphocreatine, 1 mM ATP, 3 µg/ml oligomycin and 3 µg/ml antimycin. After incubation for 3 h at 17 °C, 3 µM fluo-3 was added. Fluo-3 fluorescence was monitored at 490 nm

excitation and 535 nm emission in a 250  $\mu$ l cuvette at 17 °C with a circulating water bath and continuously mixed with a magnetic stirring bar, using an Hitachi spectrofluorimeter (F-2000). Addition of stock solutions of various substances did not exceed 2% of the total homogenate volume in the cuvette. This method is very sensitive and specific for the detection of cADPR and NAADP when homologous desensitization and specific inhibitors are used [6,31].

#### HPLC analysis of nucleotides

The synthesis of NAADP and cADPR by mammalian tissue extracts was verified by HPLC analysis, performed by anion-exchange chromatography using a AG MP-1 column (Bio-Rad) eluted with a non-linear gradient of trifluoroacetic acid, as described previously [6,31]. The nucleotides were detected by UV absorption at 254 nm. The authenticity of the NAADP and cADPR produced were confirmed by co-elution with NAADP and cADPR standards and by the sea urchin egg homogenate bioassay.

#### Immunoprecipitation of CD38

Plasma-membrane extracts, solubilized in 1% (w/v) CHAPS, were incubated for 3 h at 4 °C with goat polyclonal anti-CD38 sera, raised against the C-terminal 20 amino acids of murine CD38 (SC7049; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or a non-specific IgG<sub>1</sub> diluted in PBS containing 1 mg/ml BSA, as described previously [33]. After incubation, Protein A–Cellulofine (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) was added for a further 3 h, and the reaction mixture was centrifuged at 3000 *g* for 15 min at 4 °C. The resulting immunoprecipitates and lysate were assayed NAADP and cADPR synthesis.

#### Western blot for CD38

Samples (100  $\mu$ g) were subjected to SDS/PAGE on 10% (w/v) gels and the proteins were electroblotted on to a nitrocellulose membrane. After being blocked with 5% (w/v) non-fat milk, the membrane was probed with goat polyclonal anti-CD38 antibodies (Santa Cruz). The immunoreactive bands were detected by using horseradish peroxidase-conjugated anti-(goat IgG) secondary antibody and an enhanced chemiluminescence detection system (ECL<sup>®</sup>; Amersham Biosciences, Arlington Heights, IL, U.S.A.). Bands were scanned by using a PhosphorImager (Bio-Rad).

#### Assay of adenylate cyclase

Adenylate cyclase activity was determined in the presence of 10  $\mu$ M forskolin as described previously [31]. The extracts were incubated with 0.1 mM ATP, and the content of cAMP was determined at the beginning and end of the incubation period by RIA.

#### Materials

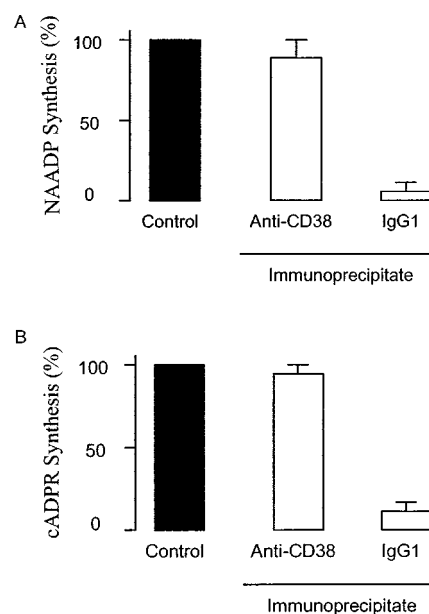
*L. pictus* and *Aplysia californica* were obtained from Marinus Inc. (Long Beach, CA, U.S.A.). Fluo-3 was purchased from Molecular Probes (Eugene, OR, U.S.A.), and IP<sub>3</sub>, ryanodine, oligomycin and antimycin were from Calbiochem (La Jolla, CA, U.S.A.). All other reagents were supplied from Sigma Co. (St. Louis, MO, U.S.A.) and of the highest-purity grade available.

The reported experiments were repeated at least three to six times.

## RESULTS AND DISCUSSION

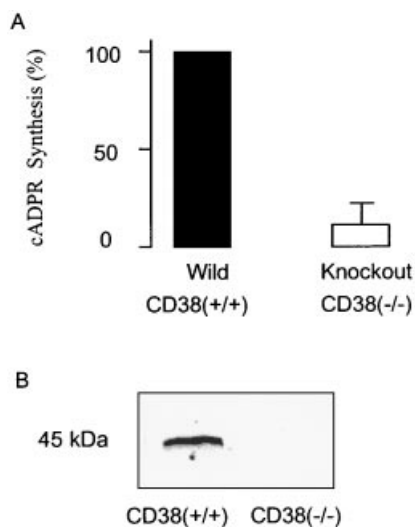
### Synthesis of NAADP and cADPR in rat tissues

In the present study, the synthesis of NAADP and cADPR was determined in several mammalian tissues. As described previously [19,20], rat tissues, including brain, liver, myocardium, spleen and lung, are able to catalyse the synthesis of both cADPR and NAADP. In contrast, kidney cortex and skeletal muscles have a very low capability of synthesizing these compounds. In fact, a linear relation was observed between the ability of tissues to catalyse NAADP and cADPR synthesis (results not shown). A ratio between the synthesis of NAADP and cADPR of  $24 \pm 4:1$  was observed in all tissues tested, with a correlation coefficient of 0.97. In view of these observations, we postulate that the same enzyme in rat tissues could catalyse the synthesis of cADPR and NAADP. Furthermore, we have also observed (results not shown) that the intracellular distributions of cADPR and NAADP synthesis were similar in several rat tissues, with the majority of the enzymic activity being present in the plasma membrane fraction and, to a lesser extent, in mitochondrial sarcoplasmic reticulum. We also explored the effect of nicotinic acid upon the synthesis of cADPR and NAADP in several rat tissues (results not shown). We have observed that in all tissues tested the half-maximal concentrations of nicotinic acid required for inhibition of cADPR synthesis and stimulation of the catalysis of NAADP were in the same range between 18–22 mM (results not shown). Taken together, these data indicate that similar enzymes catalyse the synthesis of NAADP and cADPR in rat tissues.



**Figure 1** Immunoprecipitation of NAADP-synthesizing enzyme with anti-CD38 antibody

Rat brain plasma membranes were solubilized with CHAPS and the extracts were incubated with 20  $\mu$ g of anti-CD38 sera (anti-CD38) or normal mouse IgG<sub>1</sub> (IgG1) and then mixed with Protein A–Cellulofine, as described in the Materials and methods section. After centrifugation, the immunoprecipitate and supernatant were assayed for the synthesis of NAADP (A) or cADPR (B). The data are expressed as the percentage of control before immunoprecipitation and are means  $\pm$  S.E.M. ( $n = 3$  independent experiments); 100% NAADP and cADPR synthesis correspond to  $1.2 \pm 0.1$  nmol of NAADP/min per mg of protein and  $0.04 \pm 0.002$  nmol of cADPR/min per mg of protein respectively.



**Figure 2** Synthesis of cADPR and Western-blot analyses in brain extracts from CD38(+/+) wild-type and CD38(-/-) knockout mice

cADPR synthesis was performed as described in the Materials and methods section. (A) Extracts (1 mg of protein/ml) from brain derived from CD38(+/+) wild-type and CD38(-/-) knockout mice were incubated with NAD and the synthesis of cADPR was determined by using the sea urchin egg bioassay and HPLC analyses. cADPR synthesis is expressed as the percentage of control activity and the data are presented as the mean  $\pm$  S.E.M. ( $n = 3$  independent experiments); 100% of cADPR synthesis corresponds to  $0.03 \pm 0.001$  nmol of cADPR/min per mg of protein. (B) Western-blot analysis of the tissue extracts was performed using anti-CD38 sera as described in the Materials and methods section.

To determine further whether the synthesis of NAADP and cADPR was catalysed by the enzyme CD38, we solubilized plasma membranes from several rat tissues and immunoprecipitated the CD38-like enzyme using an anti-mouse CD38 antibody. We observed that the majority of the capacity for NAADP and cADPR synthesis was co-immunoprecipitated with the CD38 enzyme (Figure 1). This observation indicates further that this protein is the major enzyme responsible for the base-exchange reaction for NAADP and cADPR synthesis in rat tissues. The immunoprecipitated enzyme was analysed by SDS/PAGE and Western blotting with a polyclonal anti-CD38 serum. Under these conditions a single protein band of approx. 45 kDa was detected. Furthermore, chromatography of the immunoprecipitate on a concanavalin A-Sepharose column provided a single fraction with both ADP-ribosyl cyclase activity and the ability to synthesize NAADP (results not shown). This fraction was analysed further by SDS/PAGE and Western blotting using the polyclonal anti-CD38 serum. A single band of approx. 45 kDa was obtained and identified as CD38 (results not shown). These observations clearly indicate that CD38, and not another co-immunoprecipitated protein, is the enzyme responsible for the synthesis of NAADP and cADPR in our preparation.

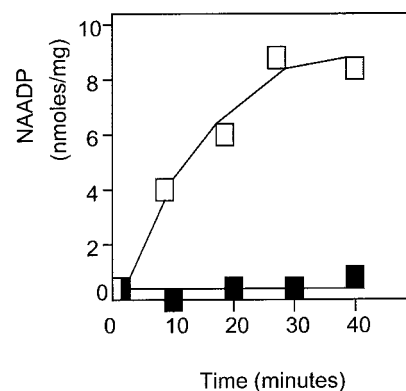
#### Synthesis of NAADP and cADPR in tissues from CD38(+/+) wild-type and CD38(-/-) knockout mice

Recently, CD38(-/-) knockout mice were generated [26,27], and used to evaluate the role of CD38 and cADPR in the mechanism of pancreatic muscarinic  $Ca^{2+}$  signalling [30]. In the present study, we have used CD38(-/-) knockout mice to determine the role of this enzyme in the synthesis of NAADP and cADPR.

**Table 1** Synthesis of nucleotides in CD38(+/+) wild-type or CD38(-/-) knockout mice

Synthesis of NAADP, cADPR and cAMP were measured as described in the Material and methods section. Protein extracts (1 mg/ml) from CD38(+/+) wild-type or CD38(-/-) knockout mice were used for the assay. Results are expressed as a percentage of control synthesis of nucleotides by wild-type mice. Data represent the mean of three to four different experiments. 100% synthesis of NAADP (in nmol of NAADP/min per mg of protein) corresponds to  $0.5 \pm 0.07$  in brain,  $0.48 \pm 0.02$  in liver,  $0.4 \pm 0.03$  in heart,  $0.6 \pm 0.03$  in spleen,  $0.3 \pm 0.05$  in lung and  $0.21 \pm 0.06$  in pancreas. 100% synthesis of cADPR (nmol of cADPR/min per mg of protein) corresponds to  $0.03 \pm 0.001$  in brain,  $0.028 \pm 0.001$  in liver,  $0.025 \pm 0.002$  in heart,  $0.04 \pm 0.002$  in spleen,  $0.018 \pm 0.002$  in lung and  $0.015 \pm 0.003$  in pancreas. N.D., not detected.

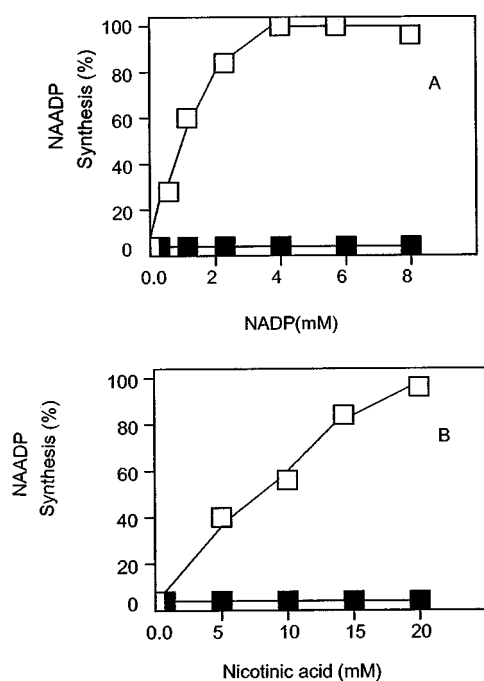
Organ	Synthesis of ...	Synthesis in CD38(-/-) knockout mice compared with CD38(+/+) wild-type mice (%)		
		NAADP	cADPR	cAMP
Brain		0.5	2	98 $\pm$ 5
Liver		N.D.	N.D.	100 $\pm$ 2
Heart		N.D.	N.D.	99 $\pm$ 6
Spleen		N.D.	N.D.	95 $\pm$ 8
Lung		0.6	N.D.	100 $\pm$ 1
Pancreas		N.D.	N.D.	100 $\pm$ 3



**Figure 3** Synthesis of NAADP in brain extracts from CD38(+/+) wild-type and CD38(-/-) knockout mice

NAADP synthesis was performed as described in the Materials and methods section. Extracts (1 mg of protein/ml) from brain derived from CD38(+/+) wild-type ( $\square$ ) and CD38(-/-) knockout ( $\blacksquare$ ) mice were incubated with 40 mM nicotinic acid and 1 mM NADP, and the time course of NAADP synthesis was determined by a combination of the sea urchin egg homogenate bioassay and HPLC analysis. Data are representative of three independent experiments.

No CD38 expression was observed by Western-blot analysis in tissues from CD38(-/-) knockout mice (Figure 2) [26,27,30]. However, a significant amount of this enzyme was detected in CD38(+/+) wild-type mice (Figure 2) [26,30]. We determined further the synthesis of both NAADP and cADPR in homogenates of CD38(+/+) wild-type and CD38(-/-) knockout mice. As described previously [26,27,30], the synthesis of cADPR was severely impaired in CD38(-/-) knockout mice (Figure 2 and Table 1). In addition, we also observed no detectable synthesis of NAADP in brain from CD38(-/-) knockout mice (Figures 3 and 4). This observation was extended further to other tissues, including liver, heart, spleen, lung, and pancreas (Table 1). In contrast with the synthesis of both cADPR and NAADP, which were almost abolished in the absence of CD38,



**Figure 4** Dose-dependent effect of NADP and nicotinic acid on NAADP synthesis from brain extracts of CD38(+/+) wild-type and CD38(-/-) knockout mice

NAADP synthesis was performed as described in the Materials and methods section. Extracts (1 mg of protein/ml) from brain derived from CD38(+/+) wild-type (□) and CD38(-/-) knockout (■) mice were incubated with nicotinic acid and NADP. (A) Extracts were incubated with 7 mM nicotinic acid and different concentrations of NADP. (B) Extracts were incubated with 1 mM NADP and different concentrations of nicotinic acid. NAADP synthesis was determined after different incubation times and the initial velocity calculated. Results are expressed as percentage of control maximal synthesis of NAADP and are representative of three independent experiments.

the synthesis of cAMP was not affected by the lack of CD38 (Table 1).

We also determined the role of CD38 on NAADP synthesis in several intracellular organelles, including microsomal fraction, mitochondria and nuclei. We found significant NAADP synthesis in plasma membrane ( $1.6 \pm 0.2$  nmol of NAADP/min per mg of protein), mitochondria ( $0.2 \pm 0.04$  nmol of NAADP/min per mg of protein), microsomal fraction ( $0.13 \pm 0.03$  nmol of NAADP/min per mg of protein) and nuclei ( $0.4 \pm 0.03$  nmol of NAADP/min per mg of protein) derived from CD38(+/+) wild-type mouse brain, with the majority of the activity present in the plasma membrane fraction. However, no detectable activity for the synthesis of NAADP was observed in any of the organelles prepared from CD38(-/-) knockout mice. Furthermore, using Western-blot analysis, we were able to detect CD38 immunoreactive bands in all preparations from CD38(+/+) wild-type, but not from CD38(-/-) knockout mice (results not shown). This observation indicates that CD38 may be the enzyme involved in NAADP synthesis, not only in plasma membrane, but also in intracellular membranes. This observation may be of major importance, since the catalytic site of CD38 on the plasma membrane appears to be facing the extracellular surface [3]. This topological paradox has been extensively discussed for cADPR synthesis in mammalian cells [3]. However, the intracellular distribution of CD38 has been described before [3]. CD38-like enzymes have been described in mitochondria, nuclei and endoplasmic reticulum [31,32,34]. In fact, in rat brain, CD38

**Table 2** Synthesis of NAADP and  $Ca^{2+}$ -releasing activity in mouse tissues

$Ca^{2+}$ -releasing activity was determined in microsomes prepared from CD38(+/+) wild-type mice and was initiated by addition of  $10 \mu\text{M}$  NAADP, as described in the Materials and methods section. N.D., not detected.

Tissue	Synthesis of NAADP (nmol of NAADP/min per mg of protein)	$Ca^{2+}$ -releasing activity (pmol/min per mg)
Brain	$0.50 \pm 0.07$	$350 \pm 28$
Liver	$0.48 \pm 0.02$	N.D.
Heart	$0.40 \pm 0.03$	$310 \pm 52$
Spleen	$0.60 \pm 0.03$	$204 \pm 30$
Lung	$0.30 \pm 0.05$	N.D.
Pancreas	$0.21 \pm 0.06$	$170 \pm 20$
Skeletal muscle	$0.07 \pm 0.03$	N.D.
Kidney	$0.10 \pm 0.04$	N.D.

immunoreactivity has been observed by ultrastructural analyses in rough endoplasmic reticulum, small vesicles, mitochondria, synaptic vesicles and the nuclear envelope [34]. The intracellular fraction of CD38 may in fact be more important for cADPR and NAADP signalling pathways, since the site of action of these compounds appears to be intracellular. However, it is important to point out that extracellular actions of cADPR have been described [3]. Thus it is possible that NAADP may also have some extracellular actions, which need further investigation. In view of results presented in this study, we concluded that CD38 is the major enzyme involved in the synthesis of NAADP by the base-exchange reaction in several organelles of mammalian tissues.

Theoretically, the synthesis of NAADP can be accomplished by three different pathways, namely: (1) deamination of NADP; (2) phosphorylation of endogenous NAAD; and (3) by the base-exchange reaction as described in the present study. To date, the base-exchange reaction is the only pathway described for NAADP synthesis in biological systems [19,20]. This reaction requires high concentrations of nicotinic acid that may not be present *in vivo*. Furthermore, the optimal pH for this reaction is outside the physiological range [19]. However, compartmentalization of nicotinic acid and NADP into an acid environment could provide a possible milieu for NAADP synthesis of *in vivo*. However, further experimental data are necessary to determine whether synthesis of NAADP by the base-exchange reaction can be performed *in vivo*. We have shown previously [18] that the NAADP-induced  $Ca^{2+}$ -releasing activity does not correlate with the activity of the synthesis of NAADP in cultured cells. It is possible that cultured cells may lose some of the properties observed *in vivo*. In the present study, we found NAADP synthesis, produced by the base-exchange reaction, in all of the tissues with the capacity for NAADP-induced  $Ca^{2+}$  release (Table 2). However, no clear correlation was observed between the activity of NAADP synthesis and  $Ca^{2+}$  release. It is noteworthy that we observed a very low capacity for NAADP synthesis and  $Ca^{2+}$ -releasing activity in kidney and skeletal muscle (Table 2). These rather indirect relationships should be interpreted with caution, as the role of CD38 in the NAADP system it is not clear to date. Furthermore, whether NAADP is a true intracellular signalling molecule still remains to be determined. Nevertheless, in the present study we have shown for the first time that CD38 is the major enzyme responsible for the base-exchange reaction in several rat tissues. This observation raises the possibility of a converging signalling pathway where two distinct intracellular

Ca<sup>2+</sup> signal molecules, namely NAADP and cADPR, could be synthesized by the same enzyme.

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