Nicotinic Acid Adenine Dinucleotide Phosphate: A New Ca^{2+} Releasing Agent in Kidney

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Abstract. Nicotinic acid adenine dinucleotide phosphate (NAADP), a molecule derived from β-NADP, has been shown to trigger Ca^{2+} release from intracellular stores of invertebrate eggs and mammalian cell microsomes. NAADP-induced Ca^{2+} release occurs through a mechanism distinct from that of inositol-1,4,5-trisphosphate– or cyclic ADP-ribose–elicited Ca^{2+} release. This study investigated whether NAADP can be synthesized in rat kidney. Extracts from glomeruli, mesangial cells, and papilla have high NAADP synthetic capacities. Conversely, synthesis of NAADP in kidney cortex was almost undetectable. Furthermore, 9-*cis*-retinoic acid significantly upregulated NAADP synthesis in mesangial cells. Authenticity of NAADP biosynthesis in glomeruli was affirmed by HPLC analysis. NAADP stimulated Ca^{2+} release from mesangial cell microsomes through a pathway distinct from that of inositol-1,4,5-trisphosphate or cyclic ADP-ribose. NAADP-triggered Ca^{2+} release may play an important role in regulation of renal function.

Release of Ca^{2+} from intracellular stores is an important component of several signaling pathways (1,2). Two major mechanisms of intracellular Ca^{2+} release are well known: (*1*) the inositol-1,4,5,-trisphosphate (IP_3)-triggered Ca^{2+} release (1,2) and (*2*) the so-called Ca^{2+}-induced Ca^{2+}-release system, mediated by the ryanodine receptor/channel and activated by the endogenous nucleotide cyclic ADP-ribose (cADPR) (3–7). In addition to Ca^{2+} release induced by cADPR and IP_3, nicotinic acid adenine dinucleotide phosphate (NAADP) was recently characterized as a potent stimulator of intracellular Ca^{2+} release in invertebrates and mammalian cells (7–15). NAADP-induced Ca^{2+} release was first demonstrated in sea urchin eggs (7–9), where nanomolar concentrations trigger Ca^{2+} release via a mechanism that differs in many ways from cADPR and IP_3 (7–15).

cADPR is synthesized by the enzyme ADP-ribosyl-cyclase (ADPR-cyclase) (7). In mammalian tissues, the majority of the enzymatic activity is catalyzed by lymphocyte antigen CD38, also identified as CD38 ADPR-cyclase (16,17). Recently, we described that in kidney parenchyma and its components, only glomeruli are endowed with a high capacity for cADPR synthesis (18). Furthermore, we demonstrated that cADPR synthesis and Ca^{2+} release are present in renal mesangial cells (18,19). We have proposed that cADPR may play an important signaling role in this cell type (18,19).

In contrast with the metabolism of cADPR, far less is known about synthesis of NAADP in mammalian tissues. We previously showed that NAADP synthesis is present in several rat tissues, including brain, heart, liver, and spleen (20). However, we observed no detectable synthesis of NAADP in rat kidney (20). In view of the recent finding of NAADP-induced Ca^{2+} release in pancreatic acinar cells and brain microsomes (21,22), we investigated NAADP synthesis in extracts from major zones of rat kidney parenchyma as well as isolated glomeruli and mesangial cells. Here we report that in extracts of rat cortex, NAADP synthesis was almost undetectable. In contrast, high capacity synthesis of NAADP was found in glomeruli, mesangial cells, and papilla. This new insight provides important information about synthesis of NAADP in kidney parenchyma and reveals that glomeruli and renal papilla are endowed with a high capacity for NAADP synthesis. We suggest a role for NAADP signaling in regulation of renal function.

Materials and Methods

Tissues were harvested from adult male Sprague-Dawley rats (250 to 350 g body wt) and killed under cocktail anesthesia (50% xylazine, 20 mg/ml and 50% Ketalar, 100 mg/ml; 0.1 ml cocktail/100 g body wt). Kidney and liver were quickly dissected, chilled, and minced in ice-cold solution containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.2). Tissues were Dounce homogenized (1:4; wt/vol) using 8 to 10 strokes and centrifuged at 4000 × rpm for 10 min at 4°C. The supernatant, denoted further as extract, was collected and used for determination of enzymatic synthesis of NAADP. The protein content was measured by the method of Lowry *et al.* (23).

Preparation of Glomeruli

Glomeruli were prepared by sequential sieving as described previously (24). Briefly, rats were anesthetized by intraperitoneal injection of cocktail, and the kidneys were perfused (10 ml/min) *in situ* with prewarmed (37°C) Hanks balanced salt solution followed by ice-cold Hanks balanced salt solution. Cortical tissue was finely minced before

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passing through a stainless steel sieve (250-μm pore size). The resulting suspension was passed several times though a 22-gauge needle to ensure complete dispersion and sequentially sieved through nylon mesh of 390-, 250-, and 211-μm pore openings. The cortical suspension was then passed over a 60-μm sieve to collect the glomeruli. Purity of glomeruli was evaluated by microscopic examination, and counting preparations contained >95% glomeruli (24).

Synthesis of NAADP

NAADP was synthesized by incubating rat kidney homogenates (1 mg/ml) or membrane fraction of mesangial cells (0.3 mg/ml) with 0.2 mM β-NADP, 7 mM nicotinic acid at 37°C, in a medium containing 0.25 M sucrose and 20 mM Tris HCI (pH 6.5) for 60 min, or as specified in the Results section. The content of NAADP was determined using a sea urchin egg homogenate Ca^{2+} release bioassay (7,25).

Sea Urchin Egg Homogenate Bioassay

Homogenates from sea urchin eggs (*Lytechinus pictus*) were prepared as described previously (7). Frozen homogenates were thawed in a 17°C water bath and diluted to 1.25% in a medium containing 2 u/ml creatine kinase, 4 mM phosphocreatine, 1 mM ATP, 3 μM fluo-3. Fluo-3 fluorescence was monitored at 490 nm excitation and 535 nm emission in a 250-μl cuvette at 17°C with a circulating water bath and continuously mixed with a magnetic stirring bar, using a spectrofluorometer (F-2000; Hitachi, Ltd., Tokyo, Japan) (7,25).

Mesangial Cells

Mesangial cells were grown in cell culture from glomeruli that were isolated from adult Sprague-Dawley rats by differential sieving (26). Cell outgrowths were characterized as mesangial cells by positive immunohistochemical staining for vimentin and smooth muscle-specific actin and by negative staining for cytokeratins, factor VIII–related antigen, and common leukocyte antigen. Mesangial cells were harvested and membrane fraction was prepared as described previously (27).

Preparation of NAADP

Authentic NAADP was synthesized from β-NADP and nicotinic acid via the base-exchange enzymatic reaction catalyzed by brain NAD-glycohydrolase (7,28). The nucleotides were purified by anion-exchange HPLC (7). NAADP in all experiments was at least 97% pure as determined by HPLC analysis.

$^{45}Ca^{2+}$ Release from Microsomes

Freshly prepared microsomes (approximately 100 μg of protein) were passively loaded by incubating for 3 h at room temperature (21°C) in a medium containing 100 mM NaCl, 25 mM HEPES (pH 7.2), 1 mM $CaCl_2$, and 1 μC of $^{45}Ca^{2+}$. Release of $^{45}Ca^{2+}$ from loaded microsomes was initiated by 10-fold dilution of microsomal suspension with a buffer containing 100 mM NaCl, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 1 mM $MgCl_2$, and 25 mM HEPES (pH 7.2) (19). After 10 s, the suspension was further diluted in the same medium without or with 10 μM NAADP (final dilution 50-fold). $^{45}Ca^{2+}$ efflux was stopped 90 s after the second dilution by test agents, and the $^{45}Ca^{2+}$ retained in microsomes was separated from free $^{45}Ca^{2+}$ by a rapid filtration technique using Whatman GF/B filters. The filters were rinsed three times with a solution containing 100 mM NaCl, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 4 mM $MgCl_2$, and 25 mM HEPES (pH 7.2). The $^{45}Ca^{2+}$ retained in microsomes was determined by liquid scintillation counting.

L. pictus was obtained from Marinus, Inc. (Long Beach, CA). Fluo-3 was purchased from Molecular Probes (Eugene, OR). All other reagents, of the highest purity grade available, were supplied by Sigma Co. (St. Louis, MO).

Statistical Analyses

When appropriate, results were evaluated statistically by *t* test.

Results

NAADP Synthesis in Kidney Parenchyma

We determined NAADP synthesis in extracts of renal cortex, medulla, and papilla. We found that papilla and, to a lesser extent, medulla were able to synthesize NAADP from β-NADP and nicotinic acid (Figure 1). The rate of synthesis of NAADP in papilla is comparable to that observed in liver. In contrast, kidney cortex has a limited capacity for synthesis of NAADP (Figure 1). The amount of NAADP synthesized in cortex is near the lower limit of detection of the sea urchin egg homogenate bioassay (7). We also observed that with time the NAADP synthesized was hydrolyzed in kidney tissues more rapidly than in liver (Figure 1). This observation is compatible with our previous observation that rat kidney has a higher rate of NAADP hydrolysis than other tested tissues (20).

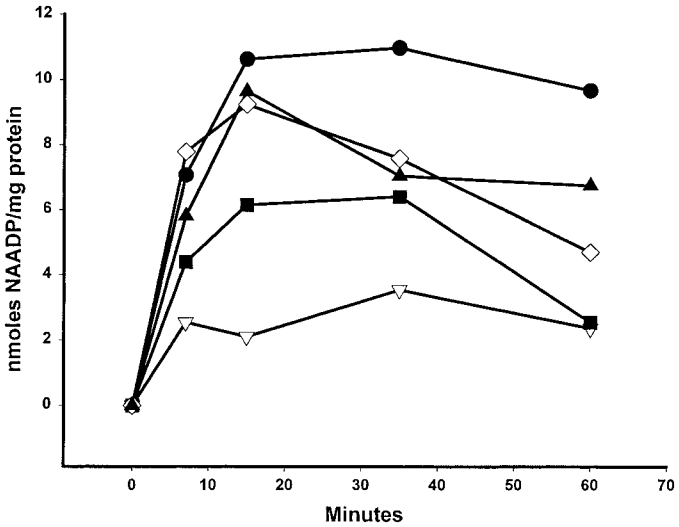

Figure 1. Biosynthesis of nicotinic acid adenine dinucleotide phosphate (NAADP) by rat kidney homogenates. Rat kidney and liver homogenates (1 mg/ml) were incubated at 37°C in a medium containing 7 mM nicotinic acid, 0.2 mM β-NADP, and 20 mM Tris HCl (pH 6.5) for 7 min, 15 min, 30 min, and 60 min. Aliquots (3 μl) of the assay media were tested for Ca^{2+} release activity using the sea urchin egg homogenate bioassay. NAADP content was determined from calibration curve of the sea urchin egg homogenate Ca^{2+} release bioassay. The calibration curve was generated by responses to known concentration of authentic purified NAADP standards. ●, liver; ◇, papilla; ■, medulla; ▽, cortex; ▲, glomeruli.

Synthesis of NAADP in Glomeruli

In view of the fact that synthesis of other signaling molecules can be distinctly localized in specific segments of the nephron (29), we determined NAADP synthesis in extracts of isolated glomeruli. Unlike extracts of renal cortex, glomeruli have a high capacity for NAADP synthesis (Figure 1). We further characterized the NAADP synthesis in glomeruli. The synthesis of NAADP was highly dependent on the concentration of precursor molecules, including β-NADP (Figure 2A) and nicotinic acid (Figure 2B). In addition, we observed a pH dependence for the synthesis of NAADP in glomeruli (Figure 3). Synthesis of NAADP in glomeruli is increased by acidification of the media; the optimal pH is approximately 4 (Figure 3). Similar pH dependence for synthesis of NAADP has been demonstrated in other systems, including sea urchin egg homogenates (25). Furthermore, the authenticity of NAADP generated by glomeruli was affirmed by HPLC analysis (Figure 4).

Synthesis of NAADP in Mesangial Cells

We further explored synthesis of NAADP in glomerular mesangial cells. We observed that mesangial cells showed a high capacity for synthesis of NAADP (Figure 5). In vascular smooth muscle cells and in LLC-PK1 cells, we previously showed that cADPR synthesis is induced by retinoic acid (27,30). Here we observed that incubation of mesangial cells with retinoic acid promotes a threefold increase in the initial rate of NAADP synthesis in these cells (Figure 5). This is the first described upregulation of NAADP synthesis in mammalian cells. We also observed a similar stimulation of NAADP synthesis by retinoic acid in LLC-PK1 and HL-60 cells (data not shown).

NAADP Induces Ca^{2+} Release from Mesangial Cell Microsomes

We also determined whether glomerular mesangial cells possess an NAADP-sensitive Ca^{2+} release mechanism. NAADP elicited Ca^{2+} release from rat mesangial cell microsomes in a dose-dependent manner, with maximal Ca^{2+} release observed after administration of 10 μM NAADP (Figure 6). Heparin, an IP_3 receptor antagonist, and 8-bromo-cADPR and ruthenium red, selective inhibitors of cADPR-mediated Ca^{2+} release through the ryanodine receptor/channel, had no effect on Ca^{2+} release activity triggered by NAADP (Figure 7). On the basis of these findings, it seems that NAADP promotes Ca^{2+} release from mesangial cell microsomes through a pathway functionally distinct from that of IP_3 or cADPR. The magnitude and time course of NAADP-induced Ca^{2+} release was similar to that of IP_3-mediated Ca^{2+} release in mesangial cell microsomes (Figure 8).

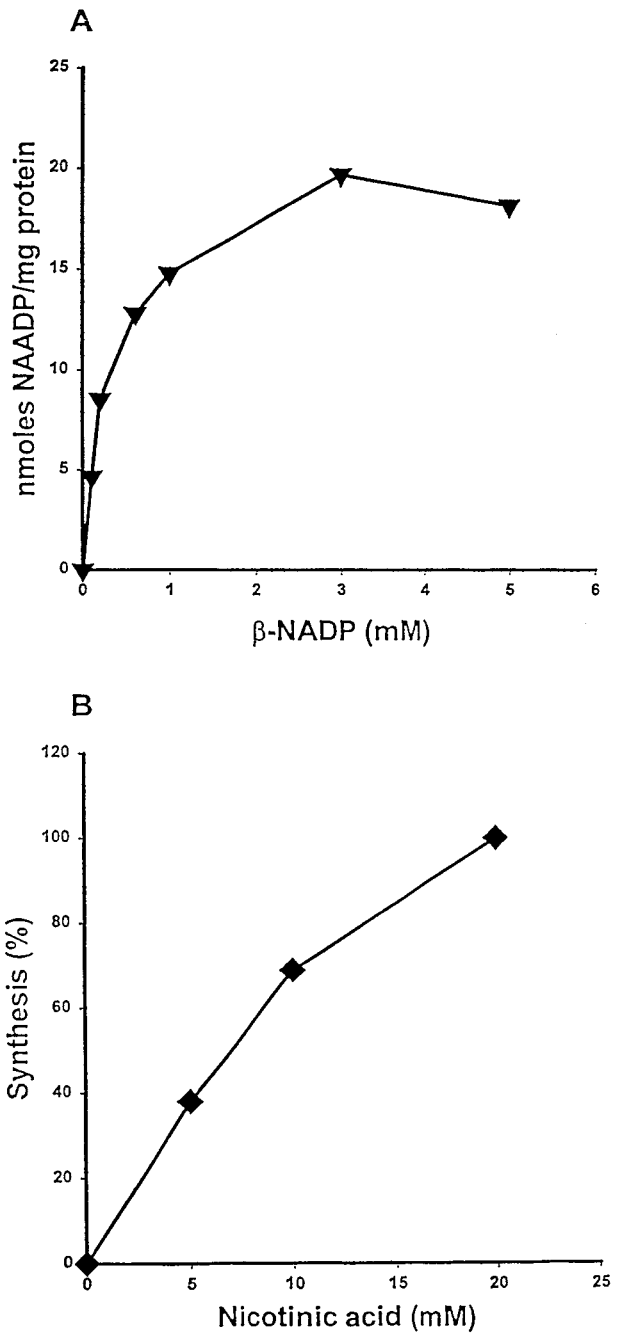

Figure 2. Dose-dependence of β-NADP and nicotinic acid on NAADP synthesis in rat kidney. (A) Dose-dependence of β-NADP. Rat glomeruli homogenate (1 mg/ml) was incubated with 7 mM nicotinic acid and different concentrations of β-NADP as substrates for 15 min, and the NAADP content was determined using the sea urchin egg homogenate bioassay. (B) Dose-dependence of nicotinic acid. Renal LLC-PK1 cell homogenate (1 mg/ml) was incubated with 0.2 mM β-NADP and different concentrations of nicotinic acid for 15 min, and the NAADP content was determined by sea urchin egg homogenate bioassay.

Discussion

It has been previously shown that NAADP is synthesized in mammalian tissues (20,25,27,30,31) and can trigger Ca^{2+} release from intracellular stores in invertebrate and vertebrate cells (7–9,21,22), suggesting that NAADP-induced Ca^{2+} release may be widespread and thus contribute to the complexity of intracellular Ca^{2+} signaling. We previously reported that some rat tissues, including brain, heart, liver, and spleen but not whole kidney, can synthesize NAADP (20). The results of the present study provide novel fundamental information re-

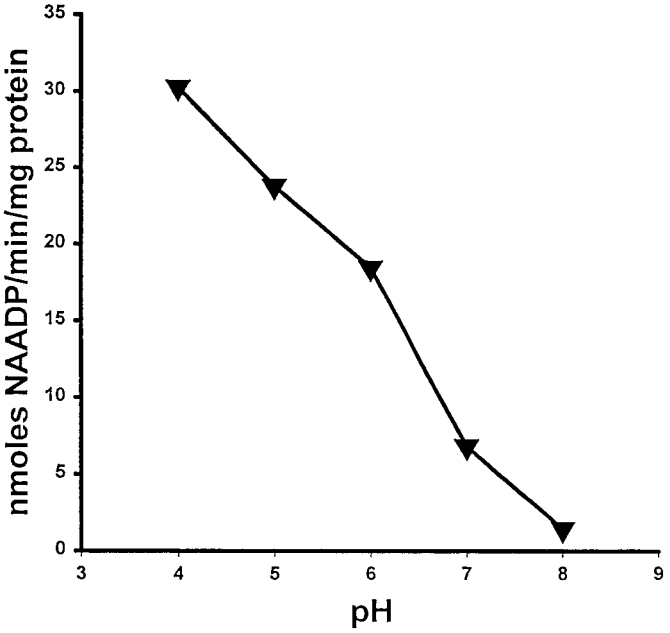

Figure 3. pH-dependence of NAADP production in rat glomeruli. Glomeruli homogenate (1 mg/ml) was incubated with 0.2 mM β-NADP and 7 mM nicotinic acid in different pHs for 15 min. NAADP content was determined by sea urchin egg homogenate bioassay.

garding the metabolism of NAADP in kidney. We found that glomeruli and papilla showed a very high capacity for NAADP synthesis, which was comparable to that observed in liver (Figure 1). In contrast, considerably less NAADP synthesis was found in extracts from renal cortex (Figure 1). Because glomeruli constitute only 5% of renal cortical mass (32), the extremely low capacity for NAADP synthesis in cortex compared with glomeruli may be due to the great diversity of cell populations in kidney. We previously reported that although limited amounts of cADPR were formed in whole kidney cortical tissue, high activity of cADPR synthesis was found in isolated glomeruli (18).

Although the role of NAADP system in kidney papilla is not known, it is important to note that regulation of intracellular Ca^{2+} is an important component in signal transduction in inner medullary collecting duct (IMCD) cells from papilla. It was recently revealed in IMCD cells from rat papilla that Ca^{2+} dependence is a unique phenomenon for stimulation of sorbitol efflux during hypotonic shock (33). It has also been reported that during hypotonic shock, intracellular Ca^{2+} is transiently elevated in IMCD cells and the increase seems to involve an initial Ca^{2+} release from intracellular stores followed by a rapid Ca^{2+} influx from the extracellular medium (34). Although the role of the NAADP-Ca^{2+} releasing system in papilla is speculative, it should be further investigated.

With respect to the site of NAADP synthesis in glomeruli, we found that extracts from mesangial cells grown in primary culture showed a significant rate of NAADP synthesis (Figure 5). Mesangial cells are specialized pericytes in glomeruli and are essential for maintenance and regulation of glomerular function (35–37). Contractility and other functions of mesangial cells are mainly regulated by Ca^{2+} released from intracellular stores (35–38). Numerous stimuli modulate the function of mesangial cells by binding to requisite receptors in the plasma membrane and via the IP_3-Ca^{2+} release pathway (38). The presence of IP_3 receptors in mesangial cells has been documented (39). More recent, we confirmed that ryanodine receptor/channel are also expressed in mesangial cells by [^{3}H]-ryanodine binding to microsomal fractions of mesangial cells and by Western blot analysis (19). In addition, we observed that the $^{45}Ca^{2+}$ release from preloaded mesangial cell

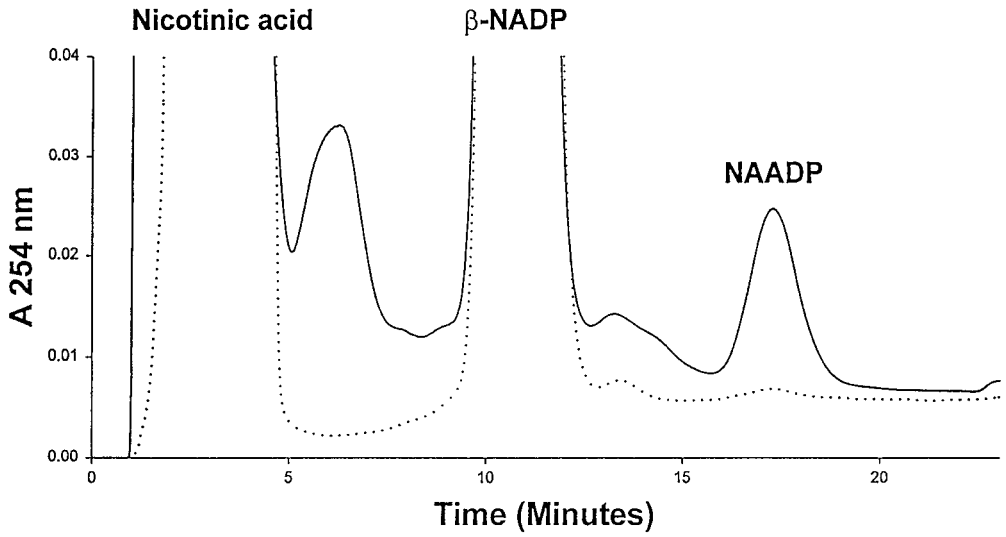

Figure 4. HPLC determination of NAADP. Rat glomeruli homogenate (1 mg/ml) was incubated with 0.2 mM β-NADP and 7 mM nicotinic acid in a 20 mM Tris HCl buffer (pH 4.0) at 37°C for 1 h. The reaction was stopped by addition of equal volume of cold acetone and centrifuged 2000 $\times$ *g* for 2 min. After acetone evaporation, the supernatant was subjected to anion-exchange HPLC analysis. The figure shows HPLC analysis of incubate at time 0 min (……) and 60 min (——) of incubation.

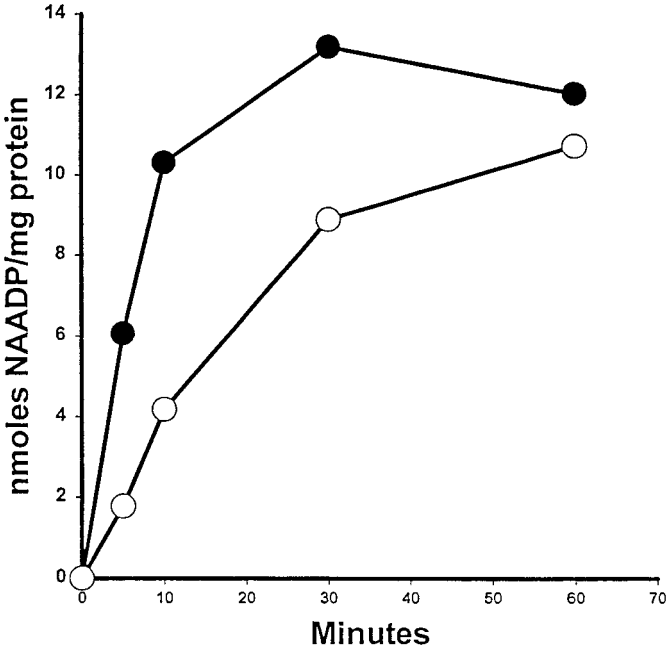

Figure 5. Synthesis of NAADP in mesangial cells. Mesangial cells were incubated with (●) or without (○) 1 μM 9-*cis* retinoic acid for 24 h, and membrane fraction was prepared as described in the Materials and Methods section. Membrane fraction of mesangial cells (0.3 mg/ml) was incubated at 37°C with 0.2 mM β-NADP and 7 mM nicotinic acid for 5 min, 10 min, 30 min, and 60 min. The amount of NAADP produced was measured by sea urchin egg homogenate Ca^{2+} release bioassay.

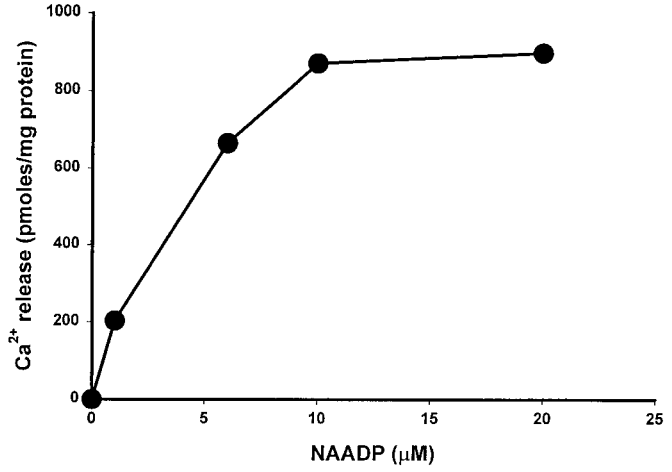

Figure 6. Dose dependency of NAADP-induced $^{45}Ca^{2+}$ release. Preloaded microsomes were incubated with different concentrations of NAADP, and $^{45}Ca^{2+}$ release was measured as described in the Materials and Methods section.

microsomes was greatly stimulated by cADPR (19) and NAADP. These data suggest that in addition to the IP_3-Ca^{2+} release pathway, cADPR- and NAADP-induced Ca^{2+} release signaling pathways may regulate the function of mesangial cells.

This study also showed that kidney extracts have a higher

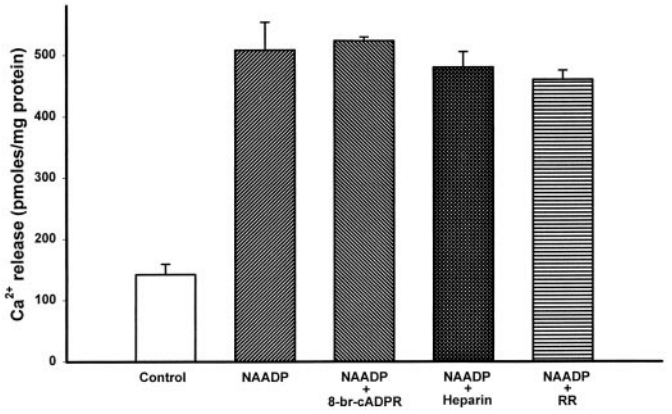

Figure 7. Effect of NAADP and inhibitors of ryanodine receptor/channel and inositol-1,4,5,-trisphosphate (IP_3) receptor on $^{45}Ca^{2+}$ release from preloaded microsomes. Ca^{2+} release was performed as described in the Materials and Methods section. □, control, no additions; ▨, 10 μM NAADP; ▧, 10 μM NAADP with 20 μM 8-br-cADPR (ryanodine channel inhibitor); ■, 10 μM NAADP with 8 μM heparin (IP_3 receptor blocker); ▤, 10 μM NAADP with ruthenium red (RR; ryanodine channel inhibitor).

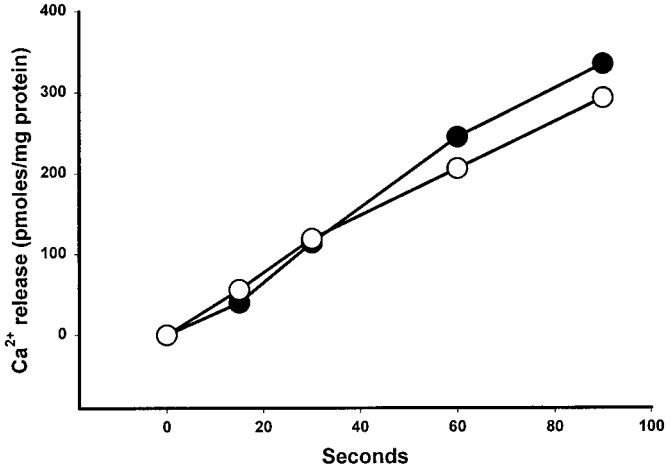

Figure 8. Time courses of $^{45}Ca^{2+}$ release induced by NAADP and IP_3. Passively loaded microsomes were incubated with 10 μM NAADP (●) or 8 μM IP_3 (○) for various time periods, and $^{45}Ca^{2+}$ release was measured as described in the Materials and Methods section.

rate of NAADP degradation than other rat tissues. Various phosphomonoesterases and phosphodiesterases may inactivate NAADP (20). Extraordinarily high inactivating activity of kidney extract may be due to high content and activity of alkaline phosphatase (40) and acid phosphatase (41) in proximal tubules.

It has been shown that the enzyme responsible for both cADPR and NAADP production has a marked pH dependence, favoring cADPR formation at a higher pH and NAADP formation at a lower pH (25). In our studies, we found that the maximal NAADP formation was observed at pH 4 (Figure 3). Although NAADP synthesis at pH 7 is relatively low compared with its synthesis at more acid pH, it was significantly higher

than cADPR synthesis at pH 7 (7 nmol NAADP/mg protein per min compared with 0.13 nmol cADPR/mg protein per min) (18), thus indicating that although the optimal condition for NAADP synthesis is in the acidic cellular compartments, NAADP production can occur in the cytosol as well.

In conclusion, our results provide strong evidence that NAADP can be synthesized and inactivated in kidney and that NAADP formation is localized in glomeruli and papilla. Furthermore, we found that NAADP induces Ca^{2+} release from mesangial cell microsomes through a pathway that is functionally distinct from that of IP_3 or cADPR. We provide preliminary evidence that NAADP production may be stimulated by extracellular stimuli, retinoic acid in particular. These data together suggest that the NAADP-Ca^{2+} release system may play a role in regulation of renal function. Future studies to determine mechanisms that underlie NAADP production will define the role of this calcium signaling system in renal pathophysiologic states.

Acknowledgments

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