Inactivation of Membrane Surface Ecto-5′-nucleotidase by Sodium Nitroprusside in C6 Glioma Cells

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Abstract. Ecto-5′-nucleotidase (NT5E), a predominant enzyme that produces extracellular adenosine from AMP, plays an important role in a variety of physiological and pathophysiological processes. This study was performed to identify agents that affect NT5E activity using C6 glioma cells. When cells were incubated with sodium nitroprusside (SNP), phorbol 12-myristate 13-acetate, forskolin, lipopolysaccharide, or interferon-γ, only SNP inhibited NT5E activity in a time- and concentration-dependent manner (IC50 = 1.2 μM). The inhibitory effect of SNP was long-lasting even after SNP washout; and its action was not mimicked by nitric oxide generating agents, 8-bromo cyclic GMP, ferricyanide, ferrocyanide, or sodium cyanide. SNP did not change NT5E mRNA level or membrane surface protein expression. Similar to SNP, Fe2+ inhibited NT5E activity, but to a lesser extent. Although Fe2+ is known to increase oxidative stress, Fe2+-mediated oxidative stress was not involved in SNP inhibition of NT5E because the inhibition of NT5E by SNP was not affected by superoxide dismutase and catalase. In contrast, addition of Zn2+, an essential metal co-factor of NT5E activity, prevented SNP from inhibiting NT5E. These results suggest that SNP disrupts a critical Zn2+-dependent enzyme activity and might be useful as a pharmacological tool for inhibiting NT5E.

Keywords: ecto-5′-nucleotidase, sodium nitroprusside, Zn2+, Fe2+, C6 glioma cell

Introduction

Ecto-5′-nucleotidase (NT5E), also known as the lymphocyte membrane antigen CD73, is a widely expressed ecto-enzyme that catalyzes the phosphohydrolysis of purine and pyrimidine monophosphates to their corresponding nucleosides. In purinergic signaling, NT5E is responsible for the formation of extracellular adenosine from AMP (1) and contributes substantially to adenosine receptor-mediated physiological responses, including modulation of neurotransmission (2, 3), cardiac function (4 – 6), platelet aggregation (7), renal function (8), and nociception (9). Previously, we demonstrated that NT5E-mediated adenosine production is coupled functionally to adenosine receptor stimulation (10, 11). In addition, NT5E is implicated in pathophysiological conditions through its control of the extracellular adenosine level, which has a protective action against ischemia-reperfusion injury in the cardiovascular (5, 6, 12) and central nervous systems (13). Furthermore, recent studies demonstrated that NT5E plays an important role in breast cancer growth, and NT5E is suggested to be a promising target for anti-cancer therapy (9, 14, 15).

Although there are several reports about the regulation of NT5E activity in many tissues, the results are controversial and the mechanism(s) underlying the regulation of NT5E activity is not clear. For example, an increase in NT5E activity was demonstrated in the ischemic heart (4, 16) and brain (17, 18), whereas NT5E activity in kidney was inhibited by hypoxic stress (19). The inactivation of renal NT5E was shown to be mediated by an increased production of nitric oxide (NO) through a mechanism independent of cyclic GMP formation (19). In cardiac tissues, however, NO was shown to increase NT5E activity through a cyclic GMP-dependent mechanism (20).

To explore factors affecting NT5E activity directly,
we investigated the effects of various agents, which stimulate intracellular signaling pathways, on NT5E activity in C6 glioma cells. We found that sodium nitroprusside (SNP) significantly reduced NT5E activity, but other NO-donors, phorbol 12-myristate 13-acetate (PMA), forskolin (FK), lipopolysaccharide (LPS), and interferon (IFN)-γ, had little effect on NT5E activity.

**Materials and Methods**

**Cell culture**

C6 glioma cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in a Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum. For the nucleotidase assay, cells were seeded in a 48-well plate and cultured until they were subconfluent. For RNA preparations and Western blot analysis, cells were cultured in 10-cm dishes.

**Measurement of NT5E activity**

NT5E activity was determined by measuring the release of inorganic phosphate (Pi) from AMP. After cells were grown to subconfluence, culture medium was changed to serum-free DMEM, and then the cells were incubated with test agents for different time periods. Cells were then washed twice with a Pi-free Krebs-Ringer’s HEPES (KRH) buffer [130 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.8 mM CaCl₂, 11.5 mM d-glucose, and 20 mM HEPES (pH 7.4)] and preincubated in a water bath at 37°C with 100 μl of KRH buffer. The reaction was started by adding 100 μl of KRH buffer containing AMP. After 10-min incubation, 100 μl of the assay solution was collected from each well and mixed with 100 μl of 10 mM EDTA to stop the AMP hydrolysis. Pi levels were determined by the malachite green–based colorimetric assay (21) as described previously (22, 23). In some experiments, NT5E activity was determined with suspended cells. Cells were harvested and washed twice with KRH buffer and suspended at 2 × 10⁶ cells/ml in fresh KRH buffer. Cells (50 – 100 μl) were then mixed with an equal volume of AMP solution. The incubation was terminated by adding EDTA to a final concentration of 10 mM. The suspension was centrifuged at 12,000 rpm for 10 s, and the cell-free supernatant was assayed for Pi as indicated above. AMP hydrolysis to adenosine was confirmed by reverse-phase HPLC on an analytical C18 column (150 × 4.6 mm; YMC Co., Ltd., Kyoto) using 50 mM NaH₂PO₄ (pH 5.5) as solvent at a flow rate of 1 ml/min as described previously (24). ATP hydrolysis was also monitored by HPLC.

**Measurement of Fe²⁺**

Fe²⁺ levels in incubation medium were monitored by a colorimetric assay using 3-(2-pyridyl)-5,6-bis(4-sulfo-phenyl)-1.2.4-triadine (PDTS), which binds to Fe²⁺ and results in an increase in absorbance at 560 nm (25). In this experiment, KRH buffer was used to avoid Fe²⁺ modification by various nutrient and vitamins in DMEM medium. Cells were incubated with KRH buffer in the presence or absence of SNP or Fe²⁺ for 6 h. The incubation medium was then collected, and cells were subjected to the NT5E assay. Aliquots of the collected medium (75 μl) were added to a 98-well plate and mixed with an equal volume of 1 mM PDTS dissolved in 0.1 M sodium acetate (pH 4.5). Developed color of the PDTS-Fe²⁺ complex was measured at 560 nm. The amount of 560 nm absorbance was proportional to the Fe²⁺ concentration from 10 to 200 μM.

**Reverse transcription–polymerase chain reaction (RT-PCR)**

Total RNA was extracted from untreated C6 glioma cells and those treated with 10 μM SNP for 12 h by the acid-guanidinethiocyanate/phenol/chloroform extraction method (26). First stranded cDNA primed by random hexamer was synthesized from 1 μg total RNA by Moloney murine leukaemia virus reverse transcriptase in a 20-μl reaction mixture and diluted to 100 μl with water. PCR amplification of NT5E was performed as described previously (10). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also amplified to monitor the quantity of cDNA template in each sample using the following primers: TGCTGAGTATGTCGTGGAGT (sense, 338 – 357) and CATACTTGGCAAGTTTCTCC (antisense, 831 – 812). The predicted lengths of PCR products of NT5E and GAPDH were 264 and 494 bp, respectively. The PCR products were subjected to 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The images were scanned and analyzed by NIH image (ver. 1.62).

**Detection of cell surface NT5E protein**

C6 glioma cells treated with or without SNP (30 μM) for 12 h were washed twice with phosphate balanced saline (PBS) and harvested. Cell surface proteins were then labeled with biotin-XX sulfosuccinimidyl ester (0.2 μg/ml) for 60 min at 4°C. After washing three times with PBS, cells were lysed in buffer containing 140 mM NaCl, 2 mM EDTA, 20 mM Tris·HCl (pH 7.4), 1% NP-40 and 1% Triton X-100, 5 μg/ml antipain/leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell debris was removed by centrifugation (10,000 × g, 5 min), and the lysates were pre-cleaned with 10 μl protein G-Sepharose (Pharmacia, Uppsala, Sweden). Biotinylated NT5E was immunoprecipitated using 2 μg/ml anti-rat NT5E mouse monoclonal antibody.
for 3 h at 4°C, followed by incubating with protein-G Sepharose at 4°C overnight. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel (11.5%) electrophoresis and transferred to a PVDF membrane. Biotinylated proteins were then visualized by streptavidin–horseradish peroxidase in combination with the ECL system. The images were scanned and analyzed by NIH image (ver. 1.62).

Data analysis
Experiments were repeated at least three times, and similar results were obtained. Statistical analyses of the data were performed by the paired Student’s t-test for two data comparison and one-way analysis of variance (ANOVA) with the Dunnett two-tailed test for multiple data comparison. For all evaluations, P values less than 0.05 were considered to be significant.

Materials
PMA, FK, LPS, superoxide dismutase, catalase, and PDTS were purchased from Sigma-Aldrich Japan (Tokyo). Isosorbide mononitrate (ISMN) was kindly provided from Toa Eiyo (Tokyo). SNP, sodium nitrite (NaNO₂), sodium nitrate (NaNO₃), sodium cyanide (NaCN), potassium ferricyanide, and potassium ferrocyanide were from Wako Pure Chemicals (Osaka). S-Nitroso-α-acetyl penicillamine (SNAP) and 1-hydroxy-2-oxo-3-(N-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC12) were from Dojindo (Kumamoto). Anti-rat NT5E mouse monoclonal antibody (5F/B9) was from BD Biosciences (Palo Alto, CA, USA). Moloney murine leukaemia virus reverse transcriptase was from Promega Corporation (Madison, WI, USA), and Taq DNA polymerase was from GE Healthcare Japan (Tokyo). Biotin-XX sulfosuccinimidyl ester was from Invitrogen-Japan (Tokyo). All other chemicals used were of reagent grade or the highest purity available.

Results
NT5E activity in C6 glioma cells
When C6 glioma cells were incubated with 250 μM AMP, Pi in the medium was increased in a time-dependent manner (Fig. 1). C6 glioma cells also hydrolyzed other purines and pyrimidine 5′-monophosphates, such as GMP and UMP, but not alkaline phosphatase substrates, such as p-nitrophenyl phosphate or β-glycerophosphate (data not shown), indicating that C6 glioma cells possess NT5E activity. In agreement with this, AMP hydrolysis by C6 glioma cells was completely inhibited by the NT5E inhibitor αβ-MeADP (100 μM), but not by the alkaline phosphatase inhibitor levamisole (1 mM). There was a linear increase in Pi release during the first 20 min of incubation. The kinetics of Pi release with different AMP concentrations for 10 min of incubation revealed that the Kₘ and Vₘₐₓ values were 38.1 ± 2.1 μM and 10.7 ± 0.3 nmol/min per 10⁶ cells (n = 3), respectively.

Effects of SNP, PMA, FK, LPS, and IFN-γ on NT5E activity
The effects of agents that stimulate different intracellular signaling mechanisms on NT5E activity were investigated. The agents tested were the guanylyl cyclase activator SNP (10 μM), protein kinase C activator PMA (0.1 μM), the adenyl cyclase activator FK (10 μM), and pro-inflammatory stimuli such as LPS (10 μg/ml) and IFN-γ (10 ng/ml). Each compound was added to the culture medium and C6 glioma cells were incubated for 0.5, 3, or 12 h before measuring AMP hydrolysis. These treatments did not affect cell viability. The NT5E activity of C6 glioma cell monolayer was 29.0 ± 1.1 nmol/mg per min (n = 6). PMA, FK, LPS, and IFN-γ had little effect on AMP hydrolysis by C6 glioma cells. In contrast, SNP markedly decreased AMP hydrolysis to 45.3 ± 2.1% (n = 6) and 18.1 ± 0.7% (n = 6) of the control at 3 and 12 h, respectively (Fig. 2).

Properties of SNP inhibition of NT5E activity
We next focused on the inhibitory effects of SNP on NT5E activity. The effect of SNP was not observed in 30 min (Fig. 3A). Thereafter, AMP hydrolysis decreased progressively in a time-dependent manner. The maximum inhibition (23.3 ± 1.2% of the control, n = 6) was obtained after 6-h incubation with SNP (Fig. 3A). Treat-
ment of cells with various concentrations of SNP (0.1 – 300 μM) for 12 h decreased AMP hydrolysis in a concentration-dependent manner with an IC50 value of 0.82 ± 0.05 μM (n = 6, Fig. 3B). After washing several times, cells were incubated with AMP (250 μM) for 20 min, and Pi released was expressed as nmol/mg protein/ min. Data are the mean ± S.E.M. (n = 6). *Significantly different from the control value at P < 0.01 by ANOVA, post-hoc Dunnett analysis.

The inhibition of NT5E by SNP was long-lasting, since NT5E activity was not recovered even after a 1-h washout period (data not shown). To examine the effect of SNP on the kinetic properties of NT5E, C6 glioma cells exposed to 10 μM SNP for 12 h were incubated with different concentrations of AMP (Fig. 4). SNP significantly decreased the Vmax value from 10.7 ± 0.3 to 2.3 ± 0.1 nmol/min per 10⁶ cells (n = 5). In contrast, the apparent Km value was only slightly affected, decreasing from 38.1 ± 2.1 to 29.9 ± 2.5 μM (n = 5). These results suggest that SNP inhibits AMP hydrolysis by NT5E in a non-competitive manner.

Effects of SNP on ATP hydrolysis in C6 glioma cells
It has been reported that C6 glioma cells express nucleotide pyrophosphatase/phosphodiesterase (NPP) (27), which hydrolyzes ATP directly to AMP without producing ADP. RT-PCR analysis revealed that the C6 glioma cells used in this study express mRNA for NPP 1, also known as PC-1, in addition to that for NT5E (data not shown). When C6 glioma cells were incubated with ATP for 10 min, ATP decreased to 62.3 ± 2.3% of the initial amount added (n = 4) and was mainly converted into adenosine (32.0 ± 2.2% of ATP added, n = 4, Fig. 5). Although pretreatment with SNP (10 μM) for 12 h had little effect on the ATP hydrolysis, adenosine formation was decreased markedly, accompanied by a parallel in-
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crease in AMP (Fig. 5). These results suggest that SNP inhibits NT5E activity without affecting NPP activity.

Effect of SNP on NT5E mRNA levels and membrane surface NT5E protein

After pretreatment of cells with 10 μM SNP for 12 h, NT5E mRNA levels were evaluated by RT-PCR. GAPDH mRNA was also amplified to normalize the PCR products, and the ratios of NT5E/GAPDH were compared. As shown in Fig. 6A, treatment with SNP did not affect NT5E mRNA levels significantly. In agreement with this, SNP did not change the membrane surface NT5E protein levels (Fig. 6B).

Effects of 8-bromo cyclic GMP, NO donors, cyanide, and iron compounds on NT5E activity

Since SNP is known to increase cyclic GMP levels through NO-mediated activation of soluble guanylyl cyclase (28), the effect of 8-bromo cyclic GMP, a lipophilic derivative of cyclic GMP on NT5E activity was examined. Treatment of C6 glioma cells with 8-bromo cyclic GMP for 24 h did not alter AMP hydrolysis (Fig. 7), suggesting that the suppression of AMP hydrolysis by SNP is not mediated by the cyclic GMP–second messenger system.

NO is known to act as oxidant molecule, causing direct nitroxyl modification of cysteine residues of proteins. Therefore, the effects of several NO donors, such as SNAP, NOC12, and ISMN were examined. Treatment with these agents for 24 h did not affect AMP hydrolysis (Fig. 7). Since SNP contains a cyanide moiety, we pretreated C6 glioma cells with 100 μM NaCN for 24 h, but this treatment did not decrease AMP hydrolysis either. These results suggest that neither NO nor CN is involved in the inhibition of NT5E activity by SNP.

Since SNP is nitrosylpentacianoferrate, we examined the effects of iron compounds on AMP hydrolysis by NT5E in C6 glioma cells. Cells were pretreated with 100 μM potassium ferricyanide or potassium ferrocyanide for 24 h, but they had no effect either (Fig. 7). Substantial inhibitory effect on NT5E activity was observed with FeCl2, but not FeCl3. FeCl2 at 10 and 100 μM decreased AMP hydrolysis to 53.2 ± 1.3% (n = 6) and 48.0 ± 3.1% (n = 6) of the control, respectively. Similar to the effects of SNP, inhibition by FeCl2 required an incubation period longer than 1 h (data not shown). However, the maximum inhibition by FeCl2 was always less than that by SNP and did not alter in the combination with the NO donor ISMN (100 μM).

Role of Fe2+ in SNP inhibition of NT5E

Since the inhibition of NT5E by Fe2+ was somewhat similar to that by SNP, we examined the contribution of Fe2+, which may be released from SNP during incubation with C6 glioma cells, to SNP inhibition of NT5E. PDTS

![Fig. 7. Effects of 8-bromo cyclic GMP (8brcGMP), NO donors, iron-containing agents, and NaCN on NT5E activity in C6 glioma cells. Cells were exposed to SNP (10 μM), 8brcGMP (1 mM), SNAP (10 μM), NOC12 (10 μM), ISMN (100 μM), FeCl2 (10 μM), FeCl3 (10 μM), ferricyanide (10 μM), ferrocyanide (10 μM), and NaCN (100 μM) for 24 h, and AMP hydrolysis was measured for 20 min with 250 μM AMP. Data are expressed as percentages of the control value presented as the mean ± S.E.M. (n = 6).](image-url)

![Fig. 6. Effect of SNP on NT5E expression in C6 glioma cells. Cells were incubated for 12 h in the absence or presence of SNP (10 μM). A: Total RNA was isolated and analyzed by RT-PCR using specific primers for NT5E and GAPDH. The RT sample was amplified 19 cycles for GAPDH and 25 cycles for NT5E. Top panels show representative results of PCR product on 1.5% agarose gel. Lower panels show relative amount of NT5E mRNA levels (mean ± S.E.M., n = 3). B: Membrane surface proteins were labeled with biotin, followed by immunoprecipitating with anti-NT5E antibody as described in Methods. Top panels show representative results of biotin-labeled NT5E protein and their relative amount are shown in the lower panels (mean ± S.E.M., n = 3).](image-url)
was used as an Fe\(^{2+}\) indicator (25). To make the result clear, we examined changes in Fe\(^{2+}\) levels in KRH buffer using higher concentrations of SNP and Fe\(^{2+}\) (100 \(\mu\)M each) after 6-h incubation with C6 glioma cells. Although SNP decreased NT5E activity to 21% of the control after 6 h, Fe\(^{2+}\) levels in the medium were lower than detectable level (Fig. 8). Fe\(^{2+}\) itself at 100 \(\mu\)M in KRH buffer was detected as around 40 \(\mu\)M after 6-h incubation. This Fe\(^{2+}\) treatment decreased NT5E activity to 38% of the control. These results suggest that a considerable amount of Fe\(^{2+}\) added is not present as its free form in KRH buffer. When PDTS assay was performed in the presence of 1 mM sodium ascorbate, which converts Fe\(^{3+}\) to Fe\(^{2+}\), Fe\(^{2+}\) levels were detected as its expected value of 103 \(\pm\) 3.2 \(\mu\)M. Even with this assay method, Fe\(^{2+}\) levels in 100 \(\mu\)M SNP solution after 6-h incubation was only slightly increased to 18 \(\mu\)M (Fig. 8). These results suggest that SNP inhibition of NT5E is not due to Fe\(^{2+}\) formation.

Fe\(^{2+}\) increases oxidative stress by catalyzing the formation of hydroxyl radical from hydrogen peroxide (H\(_2\)O\(_2\)). However, H\(_2\)O\(_2\) (100 \(\mu\)M) had little effect on NT5E activity by itself and did not alter the inhibition of NT5E by SNP (Fig. 9). Furthermore, inhibition of NT5E by SNP was not prevented by catalase (1000 U/ml) and its combination with superoxide dismutase (1000 U/ml, Fig. 8). These results indicate that oxidative modification of NT5E activity is not involved in the inhibition by SNP or Fe\(^{2+}\).

Effects of Zinc on SNP inhibition of NT5E

NT5E has been shown to contain Zn\(^{2+}\) as an essential metal ion required for enzyme activity. We examined the possibility that SNP may interfere with the role of Zn\(^{2+}\) in NT5E activity. When cells were treated with different concentrations of ZnCl\(_2\) (0.1 – 10 \(\mu\)M) for 12 h, Zn\(^{2+}\) by itself increased AMP hydrolysis in a concentration-dependent manner (Fig. 10A). Furthermore, in the presence of 1 mM sodium ascorbate, which converts Fe\(^{3+}\) to Fe\(^{2+}\), Fe\(^{2+}\) levels were detected as its expected value of 103 \(\pm\) 3.2 \(\mu\)M. Even with this assay method, Fe\(^{2+}\) levels in 100 \(\mu\)M SNP solution after 6-h incubation was only slightly increased to 18 \(\mu\)M (Fig. 8). These results suggest that SNP inhibition of NT5E is not due to Fe\(^{2+}\) formation.
of Zn$^{2+}$, the inhibitory effects of SNP on NT5E activity was diminished (Fig. 10A). When Zn$^{2+}$ was added after 6-h incubation with SNP, it restored the impaired enzyme activity in a time-dependent manner (Fig. 10B). These results suggest that SNP interferes with the role of Zn$^{2+}$ in NT5E activity.

**Discussion**

The major finding of the present study is that among several agents that affect intracellular second messenger systems, only SNP inhibited NT5E activity in C6 glioma cells. The inhibition of NT5E by SNP showed a time lag of 1 h, and the inhibition increased to more than 80% after 12 h. The inhibition of NT5E activity by SNP was concentration-dependent with an IC$_{50}$ value of around 1 $\mu$M.

SNP releases NO and stimulates cyclic GMP formation through activation of soluble guanylyl cyclase (28). This process is essential for SNP-induced vasodilatation. However, the inhibition of NT5E activity in C6 glioma cells by SNP was not mediated by NO or the cyclic GMP signaling system because various NO donors, including ISMN, SNAP, NaNO$_2$, and NOC12, did not affect NT5E activity. In addition, 8-bromo cyclic GMP, a lipophilic derivative of cyclic GMP, had no effect on NT5E activity. These results suggest that SNP inactivates NT5E in a manner that is independent of the NO–cyclic GMP signaling system.

The inhibition of NT5E activity by SNP was long-lasting, since NT5E activity was not recovered even after a 1-h washout period. The effect of SNP on the kinetic parameters of NT5E activity revealed that SNP markedly decreased $V_{\text{max}}$. Since SNP did not alter the transcription of NT5E or membrane surface immunoreactive NT5E protein level, SNP may inhibit the membrane surface enzyme directly.

There are some reports that investigated the effects of SNP and other NO generating agents on NT5E activity. However, the effects of these agents on NT5E activity seem to vary in different species and different tissues. Siegfried et al. (19) reported that NO-generating agents including SNP inhibited NT5E activity in opossum kidney cells (OK cells). The SNP inhibition of NT5E activity in OK cells was not mediated by the cGMP signaling system, but by NO through an oxidative modification of NT5E. In contrast, NO-generating agents were reported to increase NT5E activity through a cyclic GMP–dependent mechanism in rat cardiac tissue (20). On the other hand, Liu and Sok (29) demonstrated that SNP at 1 mM inactivated NT5E purified from bovine brain membrane through a mechanism by which Fe$^{2+}$ derived from SNP and thiol reagents, such as glutathione, form thiol/metal complexes, resulting in oxidative modification of the enzyme. Our results are similar to those reported with purified brain NT5E to some extent, since Fe$^{2+}$ but not Fe$^{3+}$ had an inhibitory effect on NT5E in C6 glioma cells. These results may suggest that Fe$^{2+}$ derived from SNP causes oxidative inactivation of NT5E in C6 glioma cells. However, this was not the case for the effects of SNP because we demonstrated that catalase, superoxide dismutase, and their combination were not protective against SNP inhibition of NT5E and that H$_2$O$_2$ altered neither the basal NT5E activity nor inhibition of NT5E activity by SNP. Furthermore, the inhibition of NT5E by SNP was always more potent than that by Fe$^{2+}$, whereas release of Fe$^{2+}$ from SNP was limited even after a 6-h incubation of SNP with C6 glioma cells. Taken together, it is suggested that SNP itself may have a more effective molecular structure than Fe$^{2+}$ to cause inhibition of NT5E activity. Further studies are needed to identify the chemical entities involved in the inhibition of NT5E by SNP and Fe$^{2+}$.

NT5E is known to possess a Zn$^{2+}$-binding domain in the catalytic cleft where Zn$^{2+}$ plays an essential role for enzyme activity (30). Accordingly, we demonstrated that preincubation of C6 glioma cells with Zn$^{2+}$ resulted in an increase in NT5E activity. Furthermore, Zn$^{2+}$ not only protected from SNP-induced inactivation of NT5E, but also restored the SNP-decreased enzyme activity. These results indicate that SNP may interfere with Zn$^{2+}$ at the metal binding domain, leading to an inactivation of NT5E activity.

Grobben et al. (27) showed that C6 glioma cells express NPP, an enzyme that hydrolyzes nucleotide triphosphates directly into nucleotide monophosphates. We also observed that C6 glioma cells hydrolyzed ATP and that the main product was adenosine. Pretreatment with SNP did not alter the rate of ATP hydrolysis, but it markedly impaired adenosine formation concomitant with a parallel increase in AMP. These results indicate that ATP hydrolysis by NPP is not affected by SNP, therefore indicating that SNP selectively inhibited NT5E in C6 glioma cells.

It has been demonstrated that NT5E activity is modulated in various pathophysiological conditions such as ischemia and post-ischemia reperfusion in the heart (5, 16), kidney (31), and brain (17, 18). However, the mechanisms underlying the regulation of NT5E activity are not well understood. Although cyclic AMP-dependent protein kinase (32, 33) and protein kinase C (6, 16, 34, 35) have been implicated in the regulation of NT5E activity, this is not consistent in different tissues or different cell types such as the endothelial cells (34), lymphocytes (36), and renal epithelial cells (37). In this study, we could not demonstrate any significant effects of the PKC...
accumulation, but may be due to an interruption of Zn2+-nucleotidases, such as NPP1. The inhibitory effect of ATPy without affecting ATP hydrolysis by other ecto-glioma cells that SNP is a potent inhibitor of NT5E activity.

Recent studies have shown that NT5E is upregulated in many tumor cells and involved in tumor progression by impairing antitumor T-cell responses through formation of adenosine, a potent immnosuppressive mediator (14, 15, 40). In addition, NT5E is implicated in tumor cell adhesion and migration. On the basis of these findings, inhibitors of NT5E have been proposed as potential antitumor therapeutic agents. Indeed, antitumor effects have been demonstrated with inhibition of NT5E by α,β-MeADP, monoclonal NT5E antibody, or siRNA-mediated knockdown (14, 15, 40). It may be interesting to evaluate whether SNP has any effect on tumor cell proliferation.

In conclusion, the present study demonstrates in C6 glioma cells that SNP is a potent inhibitor of NT5E activity without affecting ATP hydrolysis by other ecto-nucleotidases, such as NPP1. The inhibitory effect of SNP was not related to NO generation or cyclic GMP accumulation, but may be due to an interruption of Zn2+-dependent enzyme activity at the extracellular space. Currently, α,β-MeADP is the only available inhibitor of the NT5E. SNP may be useful as a non-nucleotide inhibitor of NT5E activity.

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