Full Paper

Inhibition by 2-Methoxy-4-ethylphenol of Ca^{2+} Influx Through Acquired and Native *N*-Methyl-D-aspartate–Receptor ChannelsRyo Fukumori^{1,†}, Noritaka Nakamichi^{1,†}, Takeshi Takarada¹, Yuki Kambe¹, Nobuyuki Matsushima^{1,2}, Nobuaki Moriguchi^{1,2}, and Yukio Yoneda^{1,*}¹Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School of Natural Science and Technology, Kanazawa, Ishikawa 920-1192, Japan²Taiko Pharmaceutical Co., Ltd., Suita, Osaka 564-0032, Japan

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Abstract. Pharmacological properties were evaluated for the antidiarrheic wood creosote ingredient 2-methoxy-4-ethylphenol (2M4EP), which was shown to be protective against neurotoxicity of *N*-methyl-D-aspartate (NMDA), to modulate Ca^{2+} influx across acquired and native NMDA receptor (NMDAR) channels. NMDA markedly increased intracellular free Ca^{2+} levels in HEK293 cells transfected with the expression vector of either NR2A or NR2B subunit together with the essential NR1 subunit vector. Further addition of dizocilpine inhibited the increase by NMDA in intracellular Ca^{2+} levels in both types of acquired NMDAR channels, while 2M4EP and the NR2B-subunit-selective antagonist ifenprodil were more effective in inhibiting the increase by NMDA in HEK293 cells expressing NR1/NR2B subunits than in those with NR1/NR2A subunits. 2M4EP significantly prevented the increased intracellular Ca^{2+} levels by NMDA in cultured rat hippocampal neurons. Brief exposure to NMDA led to a drastic decrease in cellular viability 24 h later in cultured hippocampal neurons, while 2M4EP significantly prevented the loss of cellular vitality by NMDA. Similarly, 2M4EP more efficiently protected HEK293 cells with NR1/NR2B subunits than those with NR1/NR2A subunits. These results suggest that 2M4EP may protect neurons from excitotoxicity through inhibition of Ca^{2+} influx across NMDAR channels composed of NR1/NR2B, rather than NR1/NR2A, subunits.

Keywords: antidiarrheic, *N*-methyl-D-aspartate receptor, Ca^{2+} influx, acquired channel, hippocampal neuron

Introduction

Accumulating evidence for the possible usefulness of particular natural products containing polyphenol domains for the prophylaxis as well as the therapy of different diseases relevant to oxidative stress is available in the literature. For example, catechin (1), rutin (2), green tea polyphenol (3), olive oil polyphenol (4, 5), and apple polyphenol (6) were shown to exert a protective action against osteopenia and/or osteoporosis in experimental animals. Although the proposal that these protective actions are mainly mediated by a common antioxidative property of the polyphenol against reactive oxygen spe-

cies (ROS) is the prevailing one, little attention has been paid to pharmacological properties of compounds with a single phenol moiety to date. We have previously shown the possible protective properties of major ingredients with a single phenol moiety in the traditional antidiarrheic drug wood creosote, which has been used for more than 100 years in Japan, against the cytotoxicity of oxidative stress in cultured rat calvarial osteoblasts (7) and hippocampal neurons (8). These include 2-methoxyphenol (2MP), 2-methoxy-4-methylphenol (2M4MP), and 2-methoxy-4-ethylphenol (2M4EP), which are phenolic constituents in the traditional antidiarrheic drug. Wood creosote has been often confused with the carcinogenic coal tar creosote due to the similarity of their chemical names, but they are completely different from each other with regards to the structures and properties.

Intermittent oral administration of either 2M4MP or

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2M4EP, but not 2MP, significantly prevents bone loss in ovariectomized mice *in vivo*, while both 2M4MP and 2M4EP significantly inhibit cellular maturation and differentiation of the bone-resorbing osteoclasts with concomitant protection of the bone-forming osteoblasts against oxidative stress by hydrogen peroxide (H_2O_2) *in vitro* (7). Several phenolic compounds including 2MP (also known as guaiacol), eugenol, and phenol are shown to enhance the uptake of [^3H]thymidine in the murine osteoblastic cell line MC3T3-E1 (9). 2MP is also shown to decrease the level of hydroxyl radical generated by the Fenton reaction (10). In our previous study, moreover, both 2M4MP and 2M4EP have an anti-oxidative property through scavenging hydroxyl radicals as revealed by electron spin resonance analysis (7). In the brain, oxidative stress is shown to be highly cytotoxic to neuronal (11, 12), astroglial (13), and oligodendroglial (14) cells. In fact, both 2M4MP and 2M4EP effectively prevented cell death by H_2O_2 as well as by glutamate in a concentration-dependent manner in cultured rat brain astrocytes and neurons (8). Amongst the three phenolic ingredients described above, 2M4EP is most potent in protecting cultured rat hippocampal neurons from the loss of cellular viability after exposure to glutamate (8).

In order to evaluate the mechanism underlying the protection by 2M4EP of neurons from the excitotoxicity mediated by the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR), we have attempted to determine Ca^{2+} influx across acquired NMDAR channels in HEK293 cells transfected with NMDAR-subunit expression vectors, in addition to native NMDAR channels in cultured rat hippocampal neurons.

Materials and Methods

Materials

2M4EP was purchased from Tokyo Kasei (Tokyo). Dulbecco's modified Eagle's medium (DMEM): Nutrient Mixture F-12 (1:1) was obtained from Gibco BRL (Gaithersburg, MD, USA). Versene was purchased from Invitrogen (Grand Island, NY, USA). The cell counting kit was from Dojindo (Osaka). Fluo-3 acetoxymethyl ester (AM) was provided by Molecular Probes (Eugene, OR, USA). Poly-L-lysine, cytosine arabinoside and Hoechst33342 were purchased from Sigma Chemicals (St. Louis, MO, USA). Other chemicals used were all of the highest purity commercially available.

Expression of acquired NMDAR channels

In this study, we used rat NMDAR subunits cloned into expression vectors. The plasmid constructs pcDNA1-NR2A and pcDNA3.1-NR1-1a were generous gifts from Dr. Jon W. Johnson (Department of Neuroscience, Uni-

versity of Pittsburgh, Pittsburgh, PA, USA), and pRc-CMV-NR2B plasmid was kindly given to us by Dr. David M. Lovinger (Laboratory for Integrative Neuroscience, NIAAA/NIH, Rockville, MD, USA). HEK293 cells were grown in DMEM supplemented with 5% fetal bovine serum (FBS) before transfection as described previously (15). Cells were then transfected at a 1:3 ratio with NR1-1a and either NR2A- or NR2B-subunit expression vector by the calcium phosphate co-precipitation method in minimum essential medium with 5% FBS, followed by further culture for 24 h in the presence of the NMDAR antagonist dizocilpine (MK-801) at 10 μM to protect cells from death. We also introduced the vector pEGFP-C2 (Clontech, Mountain View, CA, USA) with cloned enhanced green fluorescent protein (GFP) to monitor the transfection efficiency (16). Cells were rinsed with recording medium, followed by loading of Fluo-3 AM and subsequent exposure to 100 μM NMDA in either the presence or absence of 10 μM MK-801, 10 μM ifenprodil, and 10 to 200 μM 2M4EP for determination of intracellular free Ca^{2+} levels. Expression of corresponding proteins was invariably confirmed in cells transfected with these subunit expression vectors on Western blotting analysis.

Preparation of neuronal culture

Primary hippocampal neuronal cultures were carried out according to the method of di Porzio et al. (17), with minor modifications (18). In brief, hippocampi from 18-day-old embryonic Wistar rats were dissected and incubated with Versene at room temperature for 12 min. Cells were mechanically dissociated by using a Pasteur pipette with a fire-narrowed tip in culture medium and plated at a density of 2.0×10^5 cells/ cm^2 on plastic dishes coated with 7.5 $\mu\text{g}/\text{ml}$ poly-L-lysine in DMEM/F-12 medium with 10% FBS. After 3 days *in vitro* (DIV), medium was changed to defined medium; DMEM supplemented with 33 mM glucose, 2 mM glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5 mM HEPES, 13 mM sodium bicarbonate, 50 $\mu\text{g}/\text{ml}$ apo-transferrin, 500 ng/ml insulin, 1 pM β -estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/ml sodium selenite, and 100 μM putrescine. Cultures were treated with 10 μM cytosine arabinoside for 24 h during 2 to 3 DIV to reduce proliferating cells. Cultures were maintained for 9 DIV at 37°C in a 5% CO_2 / 95% air humidified incubator. Under these culture conditions, approximately 95% of cells were immunoreactive for the neuronal marker microtubules-associated protein-2 (MAP2) on double immunocytochemical analysis using antibodies against MAP2 and glial fibrillary acidic protein (18).

Measurement of intracellular free Ca^{2+} levels

Cultured cells were washed with recording medium containing 129 mM NaCl, 4 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 4.2 mM glucose, and 10 mM HEPES (pH 7.4) once, followed by incubation at 37°C for 50 min in recording medium containing 30 nM Pluronic F-127 and 3 μM Fluo-3 AM (19). Cultures were then washed with recording medium twice, followed by settlement for at least 1 h in the recording medium and subsequent placement in a confocal laser-scanning microscope for observation. Medium was changed once more, followed by exposure to 100 μM NMDA in either the presence or absence of 1 mM 2M4EP for determination of the fluorescence image. The calcium ionophore A23187 was then added at 10 μM to obtain the maximal fluorescence for quantitative normalization. Drugs were prepared in recording medium immediately before each use. Fluorescence images obtained with Fluo-3 were collected using an excitation wavelength of 488 nm. The parameters of illumination and detection were digitally controlled to keep the same settings throughout the experiments as described previously (19). The data obtained were subjected to quantification by normalization on the basis of fluorescence intensity in cells exposed to A23187 at 10 μM .

Determination of cell viability

Cell viability was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reduction colorimetric assays (20) with minor modifications. In brief, rat hippocampal neurons were cultured for 9 DIV, followed by replacement of culture medium with 10 mM HEPES buffer (pH 7.4) containing 129 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , and 4.2 mM glucose. Cells were then exposed to 100 μM NMDA for 1 h at 37°C in either the presence or absence of 2M4EP at concentrations of 0.1 to 1 mM, followed by further culture in routine DMEM medium for an additional 24 h and subsequent solubilization by the addition of a lysis solution containing 99.5% isopropanol and 0.04 M HCl. The amount of MTT formazan product was determined by measuring the absorbance at 550 nm on a microplate reader. Relative values were calculated as percentages over the value obtained in the control group. In addition, the background value obtained under cell-free conditions was invariably subtracted from the total value (21).

Hoechst33342 staining

Cell viability was examined by staining with the membrane permeable fluorescent dye Hoechst33342 for DNA, in association with measurement of the fluorescence of GFP. Cells were washed by recording medium and incubated with 10 $\mu\text{g}/\text{ml}$ Hoechst33342 in recording

medium for 10 min. Cells were then observed using an epifluorescent microscope (BZ-8100; Keyence, Osaka). The numbers of GFP- and Hoechst33342-positive cells were individually counted in five different visual fields chosen at random per each well, for subsequent calculation of percentages of GFP-positive cells over Hoechst33342-positive cells (22).

Data analyses

Results are all expressed as the mean $\pm$ S.E.M. and the statistical significance was determined by the two-tailed and unpaired Student's *t*-test or the one-way analysis of variance ANOVA with the Bonferroni/Dunnnett post hoc test.

Results

Effects of 2M4EP on acquired NMDAR channels

To examine whether 2M4EP directly acts on NMDAR channels, we at first artificially orchestrated functional NMDAR channels composed of different NMDAR subunits in HEK293 cells by way of transfection with either NR2A or NR2B expression vector, together with the expression vector of NR1 subunit essentially required for functional NMDAR channel orchestration. Immunocytochemical analysis clearly confirmed marked expression of immunoreactive NR1 subunit by cells with nuclei stained with Hoechst33342 at 24 h after transfection of NR1 expression vectors (data not shown). Although transient overexpression of each subunit alone did not significantly affect the percentages of GFP-positive cells over Hoechst33342-positive cells, the number of fluorescent cells was drastically decreased during the culture for 24 h in cells transfected with NR1/NR2A and NR1/NR2B expression vectors (Fig. 1A). The transfection efficiency was over 50% as measured by pEGFP-C2 vector with cloned EGFP, while introduction of NR1/NR2A and NR1/NR2B vectors led to a marked decrease in percentages of GFP-positive cells over Hoechst33342-positive cells from $52.9 \pm 1.1\%$ to $9.0 \pm 1.3\%$ for NR1/NR2A subunits and to $12.2 \pm 1.5\%$ for NR1/NR2B subunits, respectively, during culture for 24 h (Fig. 1B). However, culture with the NMDAR blocker MK-801 significantly prevented the decreases by introduction of NMDAR subunits in survival ratios of cells transfected with NR1/NR2A ($30.0 \pm 0.6\%$) and NR1/NR2B ($29.5 \pm 1.6\%$) vectors. These data suggest that glutamate in culture medium could induce cell death during culture for 24 h through activation of NMDAR in HEK293 cells artificially expressing NR1/NR2A and NR1/NR2B subunits.

Cells were then loaded with the fluorescent dye Fluo-3 for determination of intracellular free Ca^{2+} levels and

Fig. 1. Cell viability in HEK293 cells with artificial expression of NMDAR. HEK293 cells were transfected with expression vectors of each NMDAR subunit along with GFP expression vector, followed by further culture for an additional 24 h in either the presence or absence of 10 μ M MK-801 and subsequent staining with Hoechst33342 for nuclear DNA. A) Typical micrographic pictures are shown with GFP and Hoechst33342 in cells transfected with NMDAR-subunit expression vectors. B) The numbers of GFP- and Hoechst33342-positive cells were individually counted for subsequent calculation of percentages of living cells. * $P < 0.05$, significantly different from each control value in cells not cultured with MK-801.

subsequent exposure to NMDA at 100 μ M or the calcium ionophore A23187 at 10 μ M. Irrespective of the NR2 subunit expression vectors used, exposure to either NMDA or A23187 drastically increased the fluorescence intensity due to the increased intracellular free Ca^{2+} levels. The fluorescence by NMDA was quantified according to the normalization over the maximal fluorescence by A23187. Exposure to NMDA at 100 μ M immediately increased the fluorescence intensity by around 80% of that by A23187 irrespective of the expression vectors for NMDAR subunits (Fig. 2). Further addition of 2M4EP at 200 μ M resulted in similarly effective decreases in fluorescence intensity elevations by NMDA in both acquired NMDAR channels composed of NR1/NR2A (Fig. 2A) and NR1/NR2B (Fig. 2B) subunits, respectively.

In order to confirm the validity of acquired NMDAR

channels, we examined the effects of the NR2B subunit selective antagonist ifenprodil on intracellular free Ca^{2+} levels determined 5 min after the addition of NMDA. The addition of 10 μ M MK-801 drastically decreased the fluorescence intensity due to intracellular free Ca^{2+} in HEK293 cells with acquired NMDAR channels composed of NR1/NR2A and NR1/NR2B subunits (Fig. 3: A and B), whereas ifenprodil significantly inhibited the fluorescence intensity in cells expressing NR2B subunit at 10 μ M (Fig. 3B), without affecting that in cells expressing NR2A subunit (Fig. 3A). However, 2M4EP was more effective in significantly inhibiting the fluorescence intensity in a concentration-dependent manner at concentrations of 10 μ M to 1 mM in acquired NMDAR channels composed of NR1/NR2B subunits than in those of NR1/NR2A subunits (IC_{50} values; 202.9 ± 5.3 vs. 116.7 ± 7.4

Fig. 2. Effects of 2M4EP on intracellular free Ca^{2+} levels in acquired NMDAR channels. HEK293 cells were transiently transfected with expression vectors of NR2A (A) or NR2B (B) subunit, along with the essential NR1 subunit. Cells were rinsed with recording medium, followed by loading of Fluo-3 and subsequent exposure to 100 μM NMDA in either the presence or absence of 200 μM 2M4EP. Values are the mean $\pm$ S.E.M. of percentages over the maximal value obtained 5 min after the addition of 10 μM A23187 in 3 different experiments.

Fig. 3. Profiling of acquired NMDAR channels. HEK293 cells were transfected with expression vectors of NR2A (A) or NR2B (B) subunit along with the essential NR1 subunit. Cells were rinsed with recording medium, followed by loading of Fluo-3 and subsequent exposure to 100 μM NMDA in either the presence or absence of 10 μM MK-801, 10 μM ifenprodil, or 2M4EP at 10–1,000 μM for 10 min. Values are the mean $\pm$ S.E.M. of percentages over the maximal value obtained 5 min after the addition of 100 μM NMDA in 3 different experiments. * $P < 0.05$, significantly different from each control value in cells not exposed to any inhibitors.

μM , $P < 0.01$).

Effects of 2M4EP on native NMDAR channels

We next examined whether 2M4EP indeed suppresses the influx of Ca^{2+} ions through native NMDAR channels using a relatively high concentration. As shown in Fig. 4, NMDA at 100 μM markedly increased the fluorescence intensity immediately after the addition in rat hippocampal neurons cultured for 9 DIV, while prior addition of 1 mM 2M4EP significantly reduced the elevation of fluorescence intensity induced by NMDA (Fig. 4). However, fluorescence intensity was not significantly affected by 1 mM 2M4EP alone.

In order to confirm the protective property to neurons from excitotoxicity mediated by NMDAR, rat hippocampal neurons cultured for 9 DIV were exposed to 100 μM NMDA for 1 h in either the presence or absence of 2M4EP at a concentration range of 0.1 to 1 mM, followed by further culture for an additional 24 h and subsequent determination of cell viability by MTT reduction. Exposure to NMDA significantly decreased cell survival by more than 50% in cultured hippocampal neurons, whereas 2M4EP significantly prevented the loss by NMDA of cell viability at 1 mM without significantly affecting the cellular vitality alone (Fig. 5).

Fig. 4. Effects of 2M4EP on native NMDAR channels in cultured hippocampal neurons. Hippocampal neurons cultured for 9 DIV were loaded with Fluo-3, followed by exposure to 100 μ M NMDA in either the presence or absence of 2M4EP at 1 mM. Values are the mean $\pm$ S.E.M. of percentages over the maximal value obtained 5 min after the addition of 10 μ M A23187 in 3–5 different experiments. ** $P < 0.01$, significantly different from each control value in cells not exposed to 2M4EP.

Fig. 5. Effects of 2M4EP on cellular viability in cultured hippocampal neurons. Hippocampal neurons cultured for 9 DIV were exposed to 100 μ M NMDA for 1 h in either the presence or absence of 2M4EP at concentrations of 0.1 to 1 mM, followed by medium change and subsequent further culture for an additional 24 h for determination of cell viability by MTT reduction. Values are the mean $\pm$ S.E.M. in 4–6 different experiments. ** $P < 0.01$, significantly different from each control value in cells not exposed to NMDA. ## $P < 0.01$, significantly different from the values in cells not exposed to 2M4EP.

Cell death through acquired NMDAR channels

Finally, we examined whether 2M4EP protects HEK293 cells with acquired NMDAR channels from death. HEK293 cells with acquired NMDAR channels were cultured for 24 h in either the presence or absence of 2M4EP at concentrations of 10 μ M to 1 mM, followed by counting of the individual numbers of cells expressing GFP and cells stained with Hoechst33342 for subsequent calculation of percentages of living cells. Transfection

with NMDAR subunits invariably led to a marked decrease in percentages of GFP-positive cells over Hoechst33342-positive cells within culture for 24 h irrespective of subunit compositions, while the addition of 2M4EP increased percentages of living cells with GFP fluorescence in a concentration-dependent manner at 10 to 200 μ M (Fig. 6A). Quantification of these data revealed that 2M4EP significantly increased percentages of vital cells at 200 μ M in HEK293 cells expressing acquired NMDAR channels composed of NR1/NR2A ($9.0 \pm 1.3\%$ vs. $18.3 \pm 0.5\%$) and NR1/NR2B ($12.2 \pm 1.5\%$ vs. $25.1 \pm 1.5\%$) subunits (Fig. 6B). Moreover, 2M4EP was invariably more efficient in preventing the loss of cellular vitality during culture for 24 h in cells with NR1/NR2B channels than in those with NR1/NR2A channels at the concentration range used [10 μ M: $13.6 \pm 0.2\%$ vs. $19.4 \pm 0.6\%$, 200 μ M: $18.3 \pm 0.5\%$ vs. $25.1 \pm 1.6\%$, 1,000 μ M: $9.8 \pm 0.5\%$ vs. $15.3 \pm 1.4\%$]. Irrespective of subunit compositions, however, NMDA failed to significantly affect cellular viability in HEK293 cells expressing acquired NMDAR channels within culture for 24 h at 0.1 mM.

Discussion

The essential importance of the present findings is that a phenolic constituent of the traditional antidiarrheic drug wood creosote, 2M4EP, significantly prevented the decreased cell viability in cultured rat hippocampal neurons exposed to NMDA in association with inhibition of Ca^{2+} influx mediated by NMDAR channels. One possible interpretation of the cytoprotective property is that this phenolic ingredient may scavenge cytotoxic ROS

Fig. 6. Effects of 2M4EP on cell viability in HEK293 cells with acquired NMDAR channels. HEK293 cells were transfected with expression vectors of NR2A or NR2B subunit along with NR1 subunit and GFP expression vectors, followed by further culture for an additional 24 h in either the presence or absence of 2M4EP at different concentrations and subsequent staining with Hoechst33342 for nuclear DNA. The numbers of GFP- and Hoechst33342-positive cells were individually counted for subsequent calculation of percentages of living cells. Typical micrographic pictures are shown with GFP-positive cells in the panel A, while in the panel B, values are the mean $\pm$ S.E.M. in 3 different experiments for percentages of living cells. $**P < 0.01$, significantly different from each control value in cells not exposed to 2M4EP. $^{\#}P < 0.05$, significantly different from the values in cells with NR1/NR2A channel expression.

responsible for cell death as shown in cultured rat osteoblasts (7). In fact, a direct interaction is seen between certain phenolic ingredients, such as 2M4MP and 2M4EP, and hydroxyl radicals as revealed by electron spin resonance analysis (7). However, the present findings on the inhibition of upstream signals of ROS generation argue in favor of the possible involvement of a hitherto unidentified property other than an antioxidative action in the molecular mechanisms underlying the in vitro neuroprotection against excitotoxicity of NMDAR in hippocampal neurons. The finding that 2M4EP alone affected neither MTT reduction nor intracellular free Ca^{2+} levels at the highest concentration used would at least in part overcome the discouraging fact that relatively high concentrations were required for the in vitro neuroprotection compared to the routine doses ranging from 400 to 900 mg/day for wood creosote as an antidiarrheic drug in human beings. This study was originally aimed to evaluate pharmacological properties of an ingredient with a single phenol moiety in acquired and native NMDA channels in vitro, but not to elucidate the mechanisms of pharmacological actions of wood creosote in clinical use as an antidiarrheic drug. Although further evaluation of pharmacodynamics, pharmacokinetics, and toxicity of wood creosote at a high dose after oral administration in vivo (23) is undoubtedly crucial for the clinical use as an antidiarrheic drug, these comprehensive analyses seem to be beyond the scope of the present investigation. In addition to a variety of antioxidants with plural phenolic moieties such as polyphenol, by contrast, a single phenol moiety chemical was for the

first time demonstrated to be protective against NMDA neurotoxicity in cultured rat hippocampal neurons in vitro in this study. The possible correlation between antidiarrheic and neuroprotective properties of 2M4EP, however, remains to be elucidated in future studies.

It should be noted that 2M4EP markedly inhibited the increase by NMDA in intracellular free Ca^{2+} levels in cultured rat hippocampal neurons. Although exposure to NMDA could lead to the generation of ROS through the elevation of intracellular free Ca^{2+} levels after the promotion of Ca^{2+} influx in neurons (24), the present findings give rise to an idea that 2M4EP would protect neurons from neurotoxicity of NMDA through the inhibition of Ca^{2+} influx across NMDAR channels rather than the radical scavenging action. That 2M4EP significantly inhibited cell death by NMDA in cultured rat hippocampal neurons strongly supports this proposal. To our knowledge, this is the first direct demonstration of an interaction between 2M4EP and NMDAR channels in cultured rat hippocampal neurons. Calcium entry through extrasynaptic NMDAR containing an NR2B subunit is shown to be toxic to neurons, whereas Ca^{2+} influx through synaptic NMDAR containing an NR2A subunit is antiapoptotic (25). Distinct roles are proposed between synaptic and extrasynaptic NMDAR in the Ca^{2+} -dependent neurodegeneration toward excitotoxicity (26). The reason why NMDA was similarly effective in increasing intracellular free Ca^{2+} levels in HEK293 cells artificially expressing either NR1/NR2A or NR1/NR2B subunits is not fully clarified so far, while the findings obtained in this study clearly give support to an idea that 2M4EP would

rescue neurons from the excitotoxicity of glutamate mediated by NMDAR through preferential interference with Ca^{2+} influx across NMDAR channels composed of NR1/NR2B rather than NR1/NR2A subunits as seen with ifenprodil. From this standpoint, acquired channels are more useful than native channels for elucidation of the mechanisms underlying different temporal, regional, and cellular expression profiles of functional channels in a particular situation. The exact mechanism underlying the inhibition by 2M4EP of Ca^{2+} influx through acquired and native NMDA channels, however, is not fully clarified in this study.

An apparently discouraging aspect that considerably high concentrations were invariably required for the effective protection by 2M4EP is at least in part accounted for by taking into consideration the fact that cell death was artificially induced by brief exposure to 100 μM NMDA for 1 h in cultured hippocampal neurons in vitro. One possible speculation is that 2M4EP would be protective against the neurotoxicity of NMDA at a molar stoichiometry of 1:10 in native NMDAR channels in cultured hippocampal neurons, with more effective protection in acquired NMDAR channels irrespective of the subunit composition. The failure to rescue HEK293 cells with acquired NMDAR channels during culture for 24 h at 1 mM raises the possible cytotoxicity of 2M4EP itself upon chronic use at a high dose. Accordingly, 2M4EP could be a potential mother compound toward an innovative strategy for the development of a drug useful for the prophylaxis and/or treatment of degenerative brain diseases associated with cytotoxicity to neurons with less untoward properties. This traditional antidiarrheic drug wood creosote has been used without any significant untoward side effects in human beings for more than 100 years in Japan. The final conclusion should of course await the further biochemical and pharmacological profiling of antidiarrheic ingredients with a single phenolic moiety.

It thus appears that 2M4EP could prevent Ca^{2+} influx across acquired and native NMDAR, rather than non-NMDAR, channels. Future elucidation of underlying mechanisms would give us a clue for the discovery and development of a novel drug useful for the therapy and treatment of a variety of neurodegenerative and neuropsychiatric disorders associated with the neurotoxicity mediated by NMDAR in human beings.

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