Short Communication

Two Non-steroidal Anti-inflammatory Drugs, Niflumic Acid and Diclofenac, Inhibit the Human Glutamate Transporter EAAT1 Through Different Mechanisms

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Abstract. We investigated the effects of non-steroidal anti-inflammatory drugs on substrate-induced currents of L-glutamate (L-Glu) transporter EAAT1 expressed in *Xenopus laevis* oocytes. Niflumic acid (NFA) and diclofenac inhibited L-Glu-induced current through EAAT1 in a non-competitive manner. NFA produced a leftward shift in reversal potential (E_{rev}) of L-Glu-induced current and increased current amplitude at the potentials more negative than -100 mV. Diclofenac had no effects on E_{rev} and inhibited the current amplitude to the same extent at all negative potentials. These results indicate that NFA and diclofenac inhibit the L-Glu-induced EAAT1 current via different mechanisms.

[Supplementary methods and Figure: available only at <http://dx.doi.org/10.1254/jphs.09260SC>]**Keywords:** L-glutamate transporter, niflumic acid, diclofenac

L-Glutamate (L-Glu) transporters, EAATs, are the only significant mechanism for removal of L-Glu from extracellular fluid and maintenance of non-toxic concentrations. A growing body of evidence has suggested the correlation of EAATs with synaptic transmission and a variety of central nervous system (CNS) diseases (1).

Non-steroidal anti-inflammatory drugs (NSAIDs) are major anti-inflammation drugs and their effects are attributed to the inhibition of cyclooxygenase. Although NSAIDs are reported to have diverse effects on the CNS (2, 3), their effects cannot be explained only by their anti-inflammatory effects.

In recent report, fenamates, a group of NSAIDs, modulated substrate-induced current through EAATs (4, 5), suggesting a new molecular target for NSAIDs. We therefore investigated the effects of other types of NSAIDs on EAAT1.

All procedures were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. The detailed methods for expression of EAAT1 in oocytes

and electrophysiology were shown in the Supplementary Methods (available in the online version only). L-Glu was applied for 15 s with regular 30-s intervals. NSAIDs were applied from 30 s before to 5 s after the end of the application of L-Glu.

The NSAIDs were dissolved as follows: niflumic acid (NFA) (Sigma, St. Louis, MO, USA), 300 mM in DMSO; diclofenac (Wako, Osaka), 300 mM in MeOH; aspirin (Wako), 300 mM in EtOH; and indomethacin (Sigma), 100 mM in EtOH. Arachidonic acid (AA) (Calbiochem, Darmstadt, Germany) was dissolved at 100 mM in DMSO.

All data were given as the mean $\pm$ S.E.M. *P* values were obtained by statistical analysis, as noted in the figure legends.

The left traces in Fig. 1A-a illustrate inward control current produced by L-Glu (30 μ M) in *Xenopus* oocytes expressing EAAT1 at -50 mV (bold line) and -120 mV (thin line). We examined the effects of a variety of NSAIDs (Fig. 1: A and B). At -50 mV, NFA (300 μ M – 3 mM) inhibited the EAAT1 current dose-dependently. At -120 mV, NFA enhanced EAAT1 current and the effect was significant at 3 mM. *Xenopus* oocytes have endogenously Ca^{2+} -activated Cl^- channels (CaCC) and Ca^{2+} entry through voltage-dependent Ca^{2+} channels elicits a

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Fig. 1. Effects of NSAIDs on L-Glu-induced currents in EAAT1-expressing *Xenopus* oocytes. **A:** The traces of L-Glu (30 μ M)-induced inward currents in the presence or the absence of NFA (a) or diclofenac (b) at -50 mV (bold line) and -120 mV (thin line). The oocytes were held at -50 mV and hyperpolarized to -120 mV for 400 ms, every 2 s. **B:** Concentration-response relationships for various NSAIDs at -50 and -120 mV. The amplitude of EAAT1 current in the presence of the drug was normalized to that just before the application. NFA ($n = 5 - 9$), diclofenac ($n = 5$), and indomethacin ($n = 5$) significantly inhibited EAAT1 currents. Aspirin ($n = 4$) did not affect EAAT1 currents. The inhibition by NFA was always more remarkable at -50 mV than at -120 mV. $*P < 0.05$ vs. the control group. $^{\#}P < 0.05$ vs. the -50 mV group. Tukey's test following ANOVA. **C:** Concentration-response curves of EAAT1 currents at -50 mV in the absence or the presence of NFA ($n = 9$) (left) or diclofenac ($n = 8$) (right). The currents were normalized to the maximal current induced by 3 mM L-Glu. $I_{\max}$ and $K_{0.5}$ were calculated using every concentration-response trace by fitting with the following equation: $I = I_{\max}[L-Glu] / (K_{0.5} + [L-Glu])$, using Graphpad PRISM 4 for Windows. Mean $I_{\max}$ with the drug was normalized to the mean $I_{\max}$ without drug. Treatment with NFA ($n = 9$) and diclofenac ($n = 8$) resulted in a decrease in the $I_{\max}$ without affecting the $K_{0.5}$ (Student's t -test). $*P < 0.05$ vs. the control group. Tukey's test following ANOVA.

transient outward current varied by Cl^- . Although NFA is reported to inhibit CaCC (6), the contribution of CaCC to EAAT1 current may be negligible at -50 and -120 mV according to the report. Diclofenac ($100\ \mu\text{M}$ – $1\ \text{mM}$) inhibited the EAAT1 current dose-dependently. Indomethacin (100 and $300\ \mu\text{M}$) inhibited the EAAT1 current, but the effects were even weaker. In the case of diclofenac and indomethacin, the strength of the effects at -50 and -120 mV were almost the same. Aspirin ($100\ \mu\text{M}$ – $1\ \text{mM}$) had no effects. At -50 mV, co-application of NFA with varying doses of L-Glu significantly reduced the maximal current (I_{max}) to $55.1 \pm 1.8\%$ (Fig. 1C, left). The affinity for L-Glu ($K_{0.5}$) was not affected by NFA (from 50.0 ± 5.0 to $33.9 \pm 6.3\ \mu\text{M}$). Similarly, diclofenac resulted in a decrease in the I_{max} ($81.7 \pm 1.2\%$) without affecting the $K_{0.5}$ (from 27.3 ± 4.0 to $35.2 \pm 6.3\ \mu\text{M}$) in a non-competitive manner (Fig. 1C, right). Although vehicle alone had no significant effect on I_{max} and $K_{0.5}$ (data not shown), the $K_{0.5}$ value for diclofenac-vehicle was smaller than that for NFA-vehicle, which might have been attributed to the slight conformational change caused by DMSO and MeOH. Taken together, these results suggest that NFA and diclofenac modulate EAAT1 via a site different from the L-Glu recognition site.

AA is known to decrease EAAT1 current in a noncompetitive manner (7). Therefore, we compared the effects of NFA and diclofenac when co-applied with AA. The doses used for NFA and diclofenac were determined so as to obtain the equivalent effects and not to reach maximum effects (Fig. S1) (Supplementary Figure: available in the online version only). NFA and AA have an almost additive effect (Fig. 2A). Diclofenac and AA did not show an additive effect (Fig. 2B). The enhancement by AA of diclofenac's effect was significantly weaker than that of NFA's effect (Fig. 2C). These results imply that NFA and diclofenac interact with EAAT1 in different manners.

Figure 3A shows representative current–voltage curves for the EAAT1 currents in the presence or in the absence of NFA. The current value in the presence of NFA alone has been subtracted from that in the presence of NFA and L-Glu. NFA produced a significant leftward shift of reversal potential (E_{rev}) (from 28.9 ± 5.1 to 5.2 ± 5.7 mV). At the potentials more negative than -100 mV, NFA increased the current amplitude. The influence of NFA on the current amplitude was voltage-dependent (Fig. 3B). Diclofenac inhibited the current amplitude to the same extent at all negative potentials and had no effects on the E_{rev} (from 33.8 ± 9.1 to 27.5 ± 6.1 mV) (Fig. 3: C and D). These results further support the implication that NFA and diclofenac interact with EAAT1 in different manners.

The differences in voltage dependency and the effects

Fig. 2. AA differently influenced the effects of NFA and diclofenac. A and B: The percentage decrease was calculated by comparing the current amplitude induced by $30\ \mu\text{M}$ L-Glu with drugs to that induced by L-Glu alone at -50 mV. NFA and AA have an almost additive effect ($28.9 \pm 4.6\%$ decrease for NFA [$1\ \text{mM}$], $17.4 \pm 3.8\%$ decrease for AA [$100\ \mu\text{M}$], $48.3 \pm 3.4\%$ decrease for NFA + AA, $n = 4$). Although AA also enhanced the inhibitory effect of diclofenac, the enhancement was even weaker than that of NFA ($28.7 \pm 2.6\%$ decrease for diclofenac [$300\ \mu\text{M}$], $21.3 \pm 4.3\%$ decrease for AA [$100\ \mu\text{M}$], $39.3 \pm 4.0\%$ decrease for diclofenac + AA, $n = 5$). $*P < 0.05$ vs. the NSAIDs alone group. Paired t -test. C: The fold enhancement was calculated by comparing the percentage decrease by NSAIDs to that by NSAIDs with AA at -50 mV. The enhancement by AA of diclofenac's effects was significantly weaker than that of NFA's effect (1.7 ± 0.2 vs. 1.3 ± 0.1 fold; $*P < 0.05$, Student's t -test).

on E_{rev} suggest that NFA and diclofenac regulate EAAT1 via different mechanisms. The EAAT1 currents are the net result of charge movements from amino acid (aa) and ion cotransport ($\text{Na}^+ / \text{H}^+ / \text{K}^+$) (I_{aa}) and the ligand-gated Cl^- conductance (I_{Cl}) (8). NFA has been reported to en-

Fig. 3. The effect of NFA is voltage-dependent. A and C: Representative current–voltage relations for 30 μ M L-Glu in the presence or the absence of 1 mM NFA (A) and 300 μ M diclofenac (C). The current–voltage relationships were obtained with a holding potential of -50 mV and implementing 400-ms voltage jumps in 10-mV increments over the range from -120 to $+60$ mV. In the control, current values at steady-state have been subtracted from that in the presence of L-Glu. In the drug (NFA or diclofenac)–treated group, the current value in the presence of drug alone has been subtracted from that in the presence of the drug and L-Glu. NFA ($n = 10$) produced a leftward shift of E_{rev} ($P < 0.05$), whereas diclofenac ($n = 4$) had no effect (paired t -test). B and D: The relationship between the effects and the holding potential. The current in the presence of these drugs was normalized to that obtained just before the application. The effect of NFA ($n = 10$) was voltage-dependent. Diclofenac ($n = 5$) inhibited the current amplitude to the same extent at all negative potentials.

hance the L-Glu-induced EAAT4 current by activating an uncoupled H^+ conductance (5). In our preliminary data, NFA did not cause a modulation in the rate of I_{aa} and I_{Cl} , but rather modulates an uncoupled H^+ conductance of EAAT1 (unpublished observation), which may be related to the effect of NFA obtained in our study. In contrast, diclofenac altered only the current amplitude but not the E_{rev} . E_{rev} for the net current depends on the relative magnitude of I_{aa} and I_{Cl} (8). Diclofenac might have affected both I_{aa} and I_{Cl} so as not to change E_{rev} . The mechanism for the effects of diclofenac needs further investigation.

In our study, the influences on EAAT1 currents were caused by NFA, diclofenac, and indomethacin but not by aspirin. Mefenamic acid, diclofenac, and indomethacin are known to induce reproducible symptoms in patients with affective disorder (9). Aspirin has not been reported to have such side defects to date. If you take into account that GLAST (rodent EAAT1)–deficient mice showed phenotypic abnormalities related to schizophrenia (10), our results suggest that the psychiatric side effects of some NSAIDs have correlation with their effects on L-Glu transporters. Because NSAIDs have high affinity with the plasma protein, their brain delivery is restricted by the blood brain barrier (11). For example, the concentration of enantiomers of ibuprofen in cerebrospinal fluid is less than 1.5μ M after therapeutic application (12). Based on these data, the effective concentrations of NFA and diclofenac in our study are higher than that expected for therapeutic application. Habjan et al. recently indi-

cated that EAAT1 current was inhibited by NFA. The extent of the effects was application time–dependent and partially irreversible (4), raising the possibility that NFA and diclofenac modulate EAAT1 after long-term administration.

NSAIDs are widely used for patients with inflammation, fever, and pain. Our results may help understanding the mechanisms of side effects caused by some NSAIDs.

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