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# Nafamostat mesilate reversibly blocks acid-sensing ion channel currents

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**Abstract**

We electrophysiologically investigated the effects of nafamostat mesilate (NM: 6-amidino-2-naphthyl *p*-guanidinobenzoate dimethanesulfonate) and its two metabolites, 6-amidino-2-naphthol (AN) and *p*-guanidinobenzoic acid (PGBA), on three distinct types of human acid-sensing ion channels (ASICs). Acid-evoked inward currents at a holding potential of −60 mV in ASIC1a- and ASIC2a-expressing oocytes were decreased by extracellular application of NM in a concentration-dependent manner with $IC_{50}$ (inhibition constant) values of approximately 13.5 and 70.6 μM, respectively. The NM application also produced concentration-dependent inhibition of the initial-phase transient component of biphasic ASIC3 currents with an $IC_{50}$ value of approximately 2.5 μM. Application of AN showed weak blocking effects on the ASIC1a, ASIC2a, and transient ASIC3 currents with $IC_{50}$ values of approximately 1.2, 1.3, and 0.14 mM, respectively, whereas PGBA was insensitive to their currents.


*Keywords:* Acid-sensing ion channel (ASIC); Proton; Oocyte electrophysiology; Inhibition; FUT-175; Nafamostat mesilate (NM); 6-Amidino-2-naphthol (AN); *p*-Guanidinobenzoic acid (PGBA)

The acid-sensing ion channel (ASIC) family is a major branch of the degenerin/epithelial $Na^+$ channel (DEG/ENaC) superfamily. So far, four ASIC genes and six ASIC proteins—ASIC1 (ASIC1a and its splice variant ASIC1b), ASIC2 (the two splice variants ASIC2a and ASIC2b), ASIC3, and ASIC4—have been identified in mammalian organisms [1,2]. Individual ASIC proteins are subunits that associate as homomers or heteromers to form proton-gated cation channels in the central and peripheral nervous systems, and are involved in a wide range of neuronal functions such as synaptic plasticity [3], mechanosensation [4,5], sour-taste reception [6], and modulation of retinal functions [7]. The ASICs are also known to contribute to some disease states. In many acidotic conditions, such as rheumatoid arthritis and cardiac infarction, extracellular protons induce pain by opening ASICs, especially ASIC1a and ASIC3, located in nociceptors [8,9]. In the acute ischemic brain, emerging focal acidosis activates $Ca^{2+}$-permeable ASIC1a channels in neurons, contributing to $Ca^{2+}$ dependent neuronal injury [10].

Amiloride-sensitivity is a pharmacological hallmark of ASICs. However, amiloride and its derivatives are also known to block other cation channels and exchangers, such as T-type $Ca^{2+}$ channels [11] and $Na^+/H^+$ antiporters [12], as well as other member proteins belonging to the DEG/ENaC superfamily. Owing to this broad range of target molecules, the practical and experimental usage of amiloride-related compounds is relatively limited. Therefore, to further explore the physiological and pathophysiological roles of ASICs, it is quite important to identify other chemical agents that modulate their channel functions.

Nafamostat mesilate (NM; also known as FUT-175), a potent protease inhibitor, is used for the treatment of acute

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pancreatitis [13]. An undesirable aspect of the NM treatment is that NM inhibits the amiloride-sensitive $Na^+$ conductance of renal cortical collecting ducts, thereby impairing urinary $K^+$ excretion [13]. These observations strongly suggest that NM has significant affinity for the epithelial $Na^+$ channels (ENaCs), raising the possibility that NM also inhibits the currents of ASICs. To test this, the effects of NM and its two catalytically inactive metabolites, *p*-guanidinobenzoic acid (PGBA) and 6-amidino-2-naphthol (AN) [14] (Fig. 1A), on the human ASIC1a, ASIC2a, and ASIC3 channels were electrophysiologically examined in the present study.

## Materials and methods

*Molecular biology.* Full-length ASIC1a (GenBank Accession No. NM001095), ASIC2a (NM001094), and ASIC3 (NM004769) cDNAs were isolated from the human brain (for ASIC1a and ASIC2a) or trigeminal ganglia (for ASIC3) using a reverse transcription-polymerase chain reaction method. The final nucleotide sequences of the isolated clones were confirmed by DNA sequencing from both strands. The cDNAs of human α-ENaC, β-ENaC, and γ-ENaC were isolated as described previously [15].

*Oocyte electrophysiology.* The procedures were basically the same as those described previously [15]. Stage V and VI *Xenopus laevis* (*X. laevis*) oocytes were injected with 5′-capped complementary RNA transcript(s) (5 ng for homomeric ASICs or 5 ng each for the αβγ-ENaC) in a final volume of 50 nl, while control oocytes were injected with an equal volume of diethylpyrocarbonate-treated water. Two-electrode voltage-clamp recording was performed 1–3 days after injection. As the threshold pH values to activate the ASICs are close to pH 7.4 [16], we used slightly alkaline ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes, pH 8.0) as a basic superfusing medium. ASIC currents were evoked using pH 6.0 (for ASIC1a) or 4.0 (for ASIC2a and ASIC3) ND96 solutions buffered with 5 mM MES (for pH 6.0) or HOMOPIPES (for pH 4.0) instead of Hepes. The recording chamber was continuously perfused with ND96 solutions at a flow rate of 5 ml/min. All macroscopic currents were recorded at a holding potential of −60 mV. It was confirmed that water-injected oocytes generated no significant inward current at −60 mV in response to rapid drops in pH from 8.0 to 6.0 or 4.0.

*Drugs.* Amiloride obtained from Sigma Chemical (St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 500 mM as a stock solution. It was confirmed that up to 1% DMSO did not affect oocyte currents. NM, PGBA, and AN were obtained from Torii Pharmaceutical Co. (Tokyo, Japan) and Japan Tobacco Inc. (Tokyo).

*Statistics.* Pooled data are reported as means ± SE. Statistical significance between two groups was determined by Student's *t* test after one-way analysis of variance. Current values were normalized to the maximal proton-induced current of each ASIC, and the data were fitted for each experiment using the following equation: $I = a + (I_{max} - a)/(1 + (IC_{50}/[B])^n)$, where $I_{max}$ is the maximal current, $a$ is the residual current, which is insensitive to each drug, [B] is the concentration of the drug used, $IC_{50}$ is the concentration at which half-maximal blocking occurs, and $n$ is the Hill coefficient.

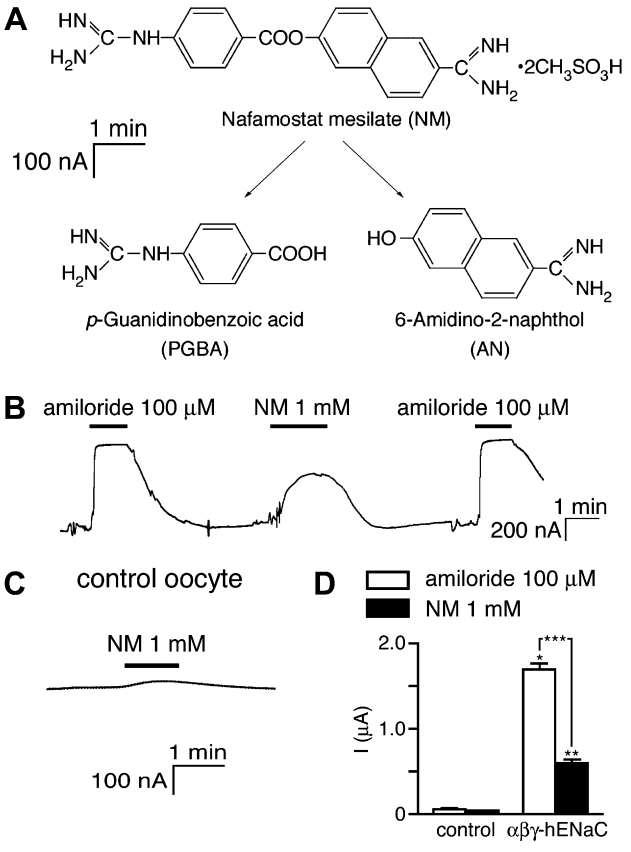


Fig. 1. Effects of nafamostat mesilate on human αβγ-ENaC (αβγ-hENaC) currents. (A) Structural formulae of nafamostat mesilate (NM), *p*-guanidinobenzoic acid (PGBA), and 6-amidino-2-naphthol (AN). (B) Representative current trace of αβγ-hENaC-expressing oocytes at a holding potential of −60 mV. (C) No significant NM (1 mM)-sensitive currents were detected in water-injected controls. (D) The effects of amiloride and NM on the oocytes are summarized. The constitutive currents of the heteromeric αβγ-hENaCs are sensitive to both 100 μM amiloride and 1 mM NM ($^*p < 0.01$ and $^{**}p < 0.01$ versus each controls). The currents were less sensitive to 1 mM NM than 100 μM amiloride ($^{***}p < 0.01$). Points represent mean values ± SE from 5 to 13 oocytes.

## Results

### *NM is less potent than amiloride at blocking constitutive currents of αβγ-ENaC*

Previous physiological experiments showed that 100 μM NM inhibited the amiloride-sensitive conductance in the apical membrane of the rabbit collecting duct cells [13]. As the amiloride-sensitive αβγ-ENaC heteromultimers are expressed in the apical membrane of the cells, controlling sodium reabsorption in the kidney [17], these findings strongly suggest that NM is able to directly act on the heteromultimeric channels by blocking their constitutive currents. To test this, we applied 1 mM NM to heterologously expressed human αβγ-ENaCs (αβγ-hENaC).

When expressed in *X. laevis* oocytes, the αβγ-hENaC induced a large inward current at a holding potential of −60 mV, and the current was mostly blocked by 100 μM amiloride (Fig. 1B). The mean amplitude of the amiloride-sensitive current in the αβγ-hENaC-expressing oocytes was 1.70 ± 0.06 μA ($n = 11$, $^*p < 0.01$ versus control of 54.4 ± 10.0 nA, $n = 7$) (Fig. 1D). The constitutive current of the channel was also sensitive to 1 mM NM, and the mean amplitude of the NM-sensitive current in the oocytes was 0.59 ± 0.05 μA ($n = 13$, $^{**}p < 0.01$ versus control of 37.4 ± 5.5 nA, $n = 5$) (Fig. 1B–D). As even 1 mM NM was less effective than 100 μM amiloride ($^{***}p < 0.01$)

(Fig. 1D), it is most likely that NM is much less potent at blocking the constitutive current than amiloride at the same concentration. On the other hand, as compared with amiloride, NM showed greater or at least equal efficacy in blocking proton-induced currents of human ASICs as described below. Therefore, we did not continue to study the effects of NM on the αβγ-hENaC any further.

*Effects of NM on proton-induced ASIC currents*

Next, the effects of NM on human ASICs were investigated using the same oocyte expression system. As the activation curves of ASIC1a and ASIC3 are very steep for pH activation [16,18], we selected pH 6.0 and 4.0 as standard stimulus pH values, respectively, which allowed us to gain a stable magnitude of the proton-induced currents of each channel. The ASIC2a channel was activated with ND96 solution of pH 4.0, which was close to its $pH_{0.5}$ value (half-point for current activation) as estimated in our expression system (data not shown).

In response to a rapid drop in pH from 8.0 to 6.0, the ASIC1a channel generated fast-rising and rapidly desensitizing inward currents that were sensitive to amiloride (Fig. 2A). The $IC_{50}$ value of amiloride for the ASIC1a currents was ~74.5 μM, and the Hill coefficient was $1.1 \pm 0.1$ ($n = 5$) (Fig. 2C). It was found that extracellular NM was also able to reduce the inward currents of the ASIC1a channel more potently than the same concentration of amiloride (Fig. 2A), and that the blockade of the currents by the NM application was reversible and concentration-dependent (Fig. 2B). The $IC_{50}$ value of NM for the inward currents was ~13.5 μM, and the Hill coefficient was $1.3 \pm 0.2$ ($n = 5$) (Fig. 2C).

The ASIC2a channel generated rapidly activating and slowly inactivating inward currents in response to an extracellular pH of 4.0, and the currents were remarkably reduced by 100 μM amiloride (Fig. 2D). The application of NM also produced reversible dose-dependent inhibition of the inward currents (Fig. 2E). In contrast to the case of the ASIC1a channel, NM seemed to be less potent than

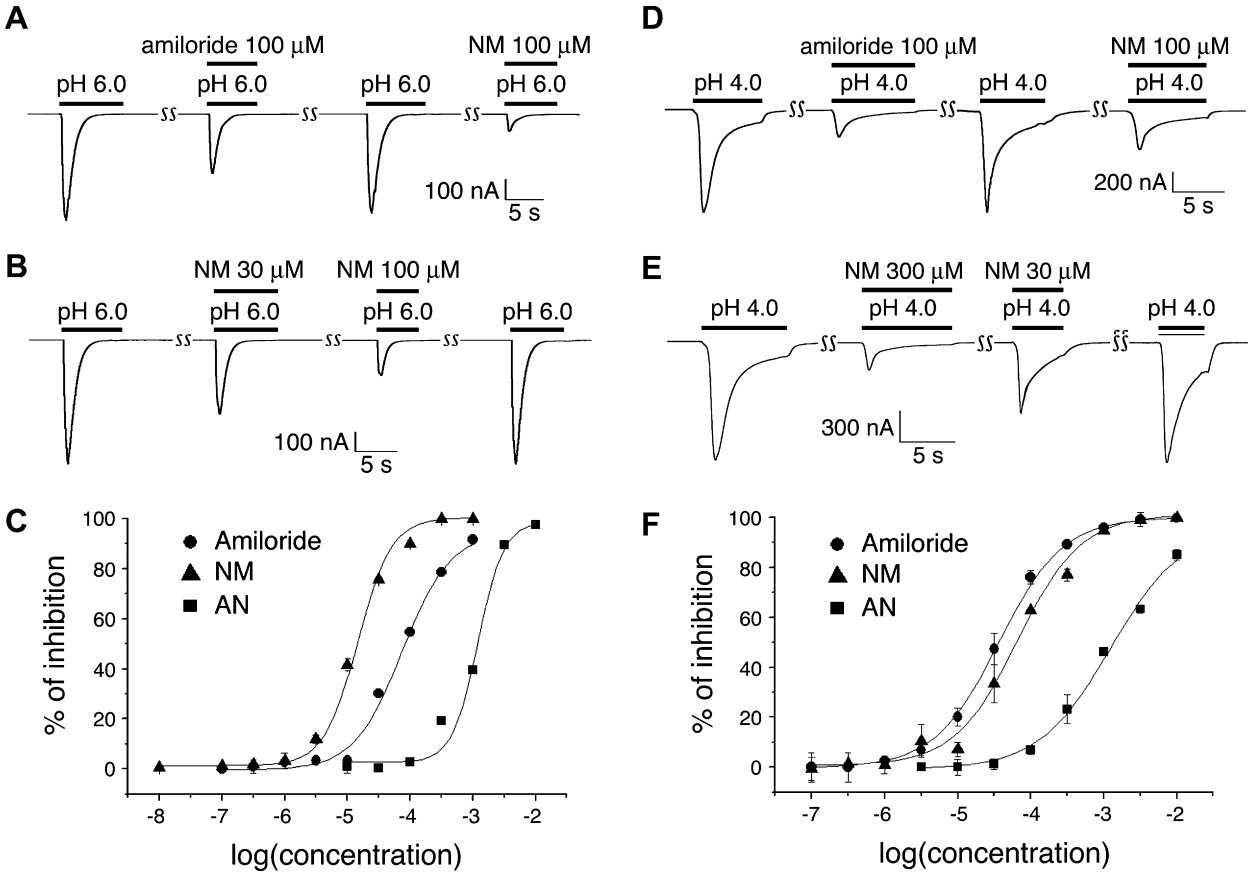


Fig. 2. Pharmacological properties of human ASIC1a and ASIC2a expressed in *X. laevis* oocytes. (A) Representative traces of acid (pH 6.0)-evoked ASIC1a currents. NM (100 μM) inhibited the currents more potently than amiloride (100 μM). (B) The NM application produced the reversible and concentration-dependent inhibition of the acid-evoked inward currents. (C) Inhibition of the macroscopic current (induced by a rapid pH change from 8.0 to 6.0) at −60 mV by amiloride, NM, and AN. Points represent mean values ± SE from 5 to 7 oocytes. (D) Representative traces of acid (pH 4.0)-evoked ASIC2a currents. NM (100 μM) inhibited the inward currents, but the application seemed not so effective as the same concentration of amiloride. (E) The NM application produced the reversible and concentration-dependent inhibition of the acid-evoked currents. (F) Inhibition of the macroscopic current (induced by a rapid pH change from 8.0 to 4.0) at −60 mV by amiloride, NM, and AN. Points represent mean values ± SE from 5 to 7 oocytes.

amiloride against the evoked currents (Fig. 2D). Dose-response relations of the ASIC2a channel to amiloride and NM revealed $IC_{50}$ values of ~34.6 μM with a Hill coefficient of $1.1 \pm 0.1$ ($n = 5$) and of ~70.6 μM with a Hill coefficient of $1.2 \pm 0.1$ ($n = 7$), respectively (Fig. 2F). However, this difference in the $IC_{50}$ values was small and statistically insignificant ($p > 0.05$).

ASIC3-expressing oocytes showed biphasic proton (pH 4.0) responses, which were characterized by fast and rapidly desensitizing currents followed by slow and sustained currents that returned to baseline on return to pH 8.0 (Fig. 3A). The peak amplitude of the transient component of the biphasic currents was remarkably decreased in the presence of extracellular amiloride, but the drug showed no reducing effect on the sustained component (Fig. 3A and B). These pharmacological profiles were in good agreement with previous observations [16,19]. The $IC_{50}$ value of amiloride for the transient currents was ~36.9 μM, and the Hill coefficient was $0.9 \pm 0.2$ ($n = 5$) (Fig. 3F). Co-application of NM with the pH 4.0 stimuli resulted in remarkable decreases in the peak amplitude of the transient ASIC3 currents, and this effect of NM was concentration-dependent and fully reversible (Fig. 3C). The $IC_{50}$ value of NM for the transient currents was ~2.5 μM, and the Hill coefficient was $0.9 \pm 0.1$ ($n = 7$) (Fig. 3F). This value was an order of magnitude lower than those required for the inhibition of the ASIC1a and ASIC2a channels. Interestingly, in contrast to amiloride, the NM application also reduced the sustained portion of the biphasic currents (Fig. 3C and D). This blockade can be seen on the condition that the enough concentrations of NM are added to bathing media in advance or at least at the same time of the extracellular acidification, because the NM application did not show any apparent effects on the sustained component when applied after the channel was activated (Fig. 3E). As far as we investigated, once the ASIC3 channel was activated, amiloride showed no effect on the sustained currents, either (Fig. 3E).

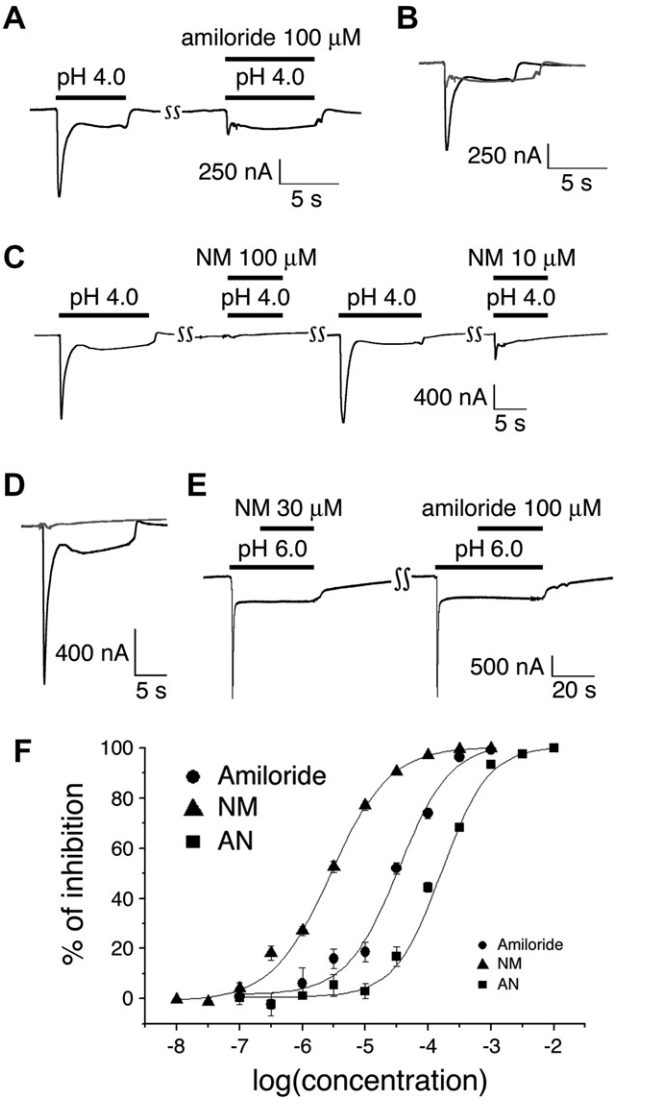


Fig. 3. Pharmacological properties of human ASIC3 expressed in *X. laevis* oocytes. (A) In response to extracellular acidification of pH 4.0, ASIC3-expressing oocytes generated fast and rapidly desensitizing currents followed by slow and sustained currents. Amiloride (100 μM) potently inhibited the transient component of the biphasic currents but showed no effect on the sustained component. (B) Amiloride block of the initial-phase transient currents in the same ASIC3-expressing oocyte. Black trace shows control currents evoked with pH 4.0, and the grey trace is steady state inhibition observed with 100 μM amiloride. (C) Extracellular NM potently inhibited not only the transient component of the biphasic currents but also the late-phase sustained currents in a concentration-dependent manner. (D) Complete inhibition of both transient and sustained currents by NM in the same ASIC3-expressing oocyte. Black trace shows control currents evoked with pH 4.0, and the grey trace is the steady state inhibition observed with 100 μM NM. (E) Extracellular NM (30 μM) and amiloride (100 μM) showed no inhibitory effect on the sustained component of the acid (pH 6.0)-evoked ASIC3 currents when applied after the channel was activated. (F) Inhibition of the initial-phase transient ASIC3 current at −60 mV by amiloride, NM, and AN. Points represent mean values ± SE from 5 to 7 oocytes.

*Effects of PGBA and AN on proton-induced ASIC currents*

We investigated the effects of PGBA and AN on the same homomeric ASICs. It was found that PGBA even as high as 1 mM displayed no blocking effect on the ASIC1a, ASIC2a, and ASIC3 channels (Fig. 4A for ASIC1a; data not shown for ASIC2a and ASIC3). On the other hand, AN apparently had inhibitory effects on the proton-induced currents of ASIC1a and ASIC2a, and on both transient and sustained components of the biphasic currents of ASIC3 (Fig. 4B–E). These effects increased in a dose-dependent manner and were fully reversible. The blockade of each channel by the AN application required much higher concentrations of the drug when compared with the amiloride or NM treatment (Figs. 2C and F, and 3F) . The $IC_{50}$ values of AN for the ASIC1a and ASIC2a channels were ~1.2 mM with a Hill coefficient of $1.6 \pm 0.2$ ($n = 7$) and ~1.3 mM with a Hill coefficient of $0.8 \pm 0.2$ ($n = 7$), respectively, and the value on the transient ASIC3 currents was ~140 μM with a Hill coefficient of $1.1 \pm 0.1$ ($n = 5$). Similar to the NM application, the extracellular AN had no effects on the sustained compo-

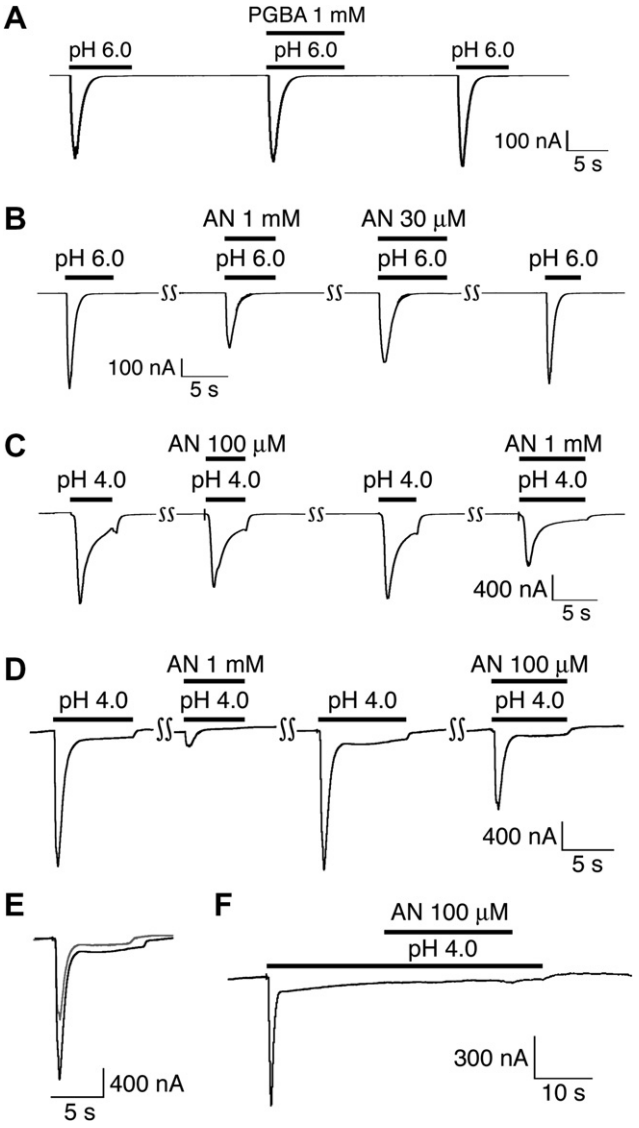


Fig. 4. Effects of PGBA and AN on the ASICs. (A) The application of 1 mM PGBA showed no apparent inhibitory effect on acid (pH 6.0)-evoked currents of ASIC1a-expressing oocytes. (B,C) AN application produced reversible and concentration-dependent inhibition of acid-evoked inward currents in ASIC1a (B)- and ASIC2a (C)-expressing oocytes. (D) Extracellular AN showed inhibitory effects on both transient and sustained components of the biphasic ASIC3 currents dose-dependently. (E) AN block of both transient and sustained currents of ASIC3 in the same oocyte. Black trace shows control currents evoked with pH 4.0, and the grey trace is the steady state inhibition observed with 100 μM AN. (F) AN (100 μM) application showed no inhibitory effect on the sustained ASIC3 currents when applied after the channel was activated with pH 4.0.

nent of the biphasic ASIC3 currents when co-applied with acidic stimuli after the channel activation (Fig. 4F).

## Discussion

There are few known ASIC blockers thus far. Amiloride (and its derivatives) and A-317567, a recently discovered small non-amiloride compound, interact with all ASIC1a, ASIC1b, ASIC2a, and ASIC3 subtypes of the channel family [20]. Psalmotoxin-1, a tarantula peptide that specifically blocks the homomeric ASIC1a channel, and APETx2, a sea anemone toxin that selectively blocks the ASIC3 channel, are subtype-specific ASIC blockers [21,22]. Our present study has added two molecules, NM and AN, to the former (non-subtype-specific) category of the blockers. Although continuous intravenous infusion of NM to patients with acute pancreatitis and disseminated intravascular coagulation occasionally causes hyperkalemia, renal ENaCs being inhibited by the drug and its two metabolites, NM has already been being used as a protease-inhibiting agent against the disorders [13,23,24]. To further discover the clinical benefits of NM, it would be important to gather information about the influences of the treatment on proposed ASIC-related matters, such as reduction in pain, from the patients.

According to our results, NM shows superior inhibitory potency against ASIC3 over amiloride. Unlike ASIC1a and ASIC2a, the ASIC3 channels are selectively expressed in peripheral sensory neurons [25], allowing us to avoid problems relevant to the CNS penetration of drugs if ASIC3 becomes a target molecule for new drug development. Interestingly, NM was able to inhibit both transient and sustained currents of the ASIC3 channel as long as the drug was applied (in advance or) at the same time of acidification. (We failed to determine the $IC_{50}$ values of NM and AN for the sustained component because the magnitude of the currents was relatively unstable.) The sustained currents of the ASIC3 and TRPV1 (transient receptor potential/vanilloid receptor subtype-1) channels in nociceptors are believed to be a fundamental cause of acidosis-linked persistent pain [2,26,27]. Although NM shows no apparent effect on the sustained ASIC3 currents when applied after the channel activation, it is likely that in local acidotic lesions, excess protons gradually, but not simultaneously, activate the population of ASIC3 channels, implying that some subpopulation of the channel may remain unaffected. Therefore, it might be interesting to develop our data eventually into the syntheses of ASIC3-specific blockers structurally related to NM, as such agents could produce analgesic effects against the pain by inhibiting the sustained component.

NM is rapidly metabolized to PGBA and AN mainly in the liver and blood, and both are excreted in the urine [23,24]. Our current pharmacological analyses on the two metabolites demonstrated that AN itself, but not PGBA, had some substantial affinity for the ASICs, although the affinity was lower than that of amiloride. These findings indicate that AN is a more selective antagonist of ASICs than NM in that AN has no inhibitory effect on some protease activities as NM has, and that AN may be a key chemical structure of NM to have the blocking effects on ASICs. While further evaluation of the mechanism underlying the interaction between NM or AN and the ASICs is required to fully understand its mode of action, these molecules may be lead compounds for drug development in the ASIC family, and at least provide new tools for the

investigation of physiology and pathophysiology related to ASICs.

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