Biophysical Journal Volume 67 December 1994 2272–2279

Potentiation of Large Conductance K_{Ca} Channels by Niflumic, Flufenamic, and Mefenamic Acids

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ABSTRACT Large conductance calcium-activated K^+ (K_{Ca}) channels are rapidly activated by niflumic acid dose-dependently and reversibly. External niflumic acid was about 5 times more potent than internal niflumic acid, and its action was characterized by an increase in the channel affinity for [Ca^{2+}], a parallel left shift of the voltage-activation curve, and a decrease of the channel long-closed states. Niflumic acid applied from the external side did not interfere with channel block by charybdotoxin, suggesting that its site of action is not at or near the charybdotoxin receptor. Accordingly, partial tetraethylammonium blockade did not interfere with channel activation by niflumic acid. Flufenamic acid and mefenamic acid also stimulated K_{Ca} channel activity and, as niflumic acid, they were more potent from the external than from the internal side. Fenamates applied from the external side displayed the following potency sequence: flufenamic acid ≈ niflumic acid ≫ mefenamic acid. These results indicate that K_{Ca} channels possess at least one fenamatereceptor whose occupancy leads to channel opening.

INTRODUCTION

Large conductance calcium-activated K channels are present in a variety of cell types (Latorre et al., 1989; McManus, 1991). In neurons they may regulate cell firing (Gola and Crest, 1993), and in smooth muscle they seem to play an important role in maintaining visceral and vascular tone (Brayden and Nelson, 1992; Suarez-Kurtz et al., 1991; Anwer et al., 1993; Khan et al., 1993). Thus, drugs that activate this type of channels from the external side or internal side (McManus et al., 1993) should be valuable pharmacological tools to modify cellular excitability, as well as to unveil mechanisms of K_{Ca} channel function.

Niflumic and flufenamic acids are nonsteroidal anti-inflammatory aromatic compounds (Hoffmann and Faure, 1966; Kohler et al., 1992) known to inhibit Cl^- conductances (White and Aylwin, 1990; Korn et al., 1991; McCarty et al., 1993) and nonselective cationic channels (Gögelein et al., 1990). Recently, a calcium-independent K current from jejunum smooth muscle and corneal epithelium has been shown to be increased by fenamates (Rae and Farrugia, 1992; Farrugia et al., 1993a, b). We now demonstrate that flufenamic ≈ niflumic ≫ mefenamic acids can activate large conductance K_{Ca} channels from the external side in a rapid and reversible manner. Part of this work has been presented in abstract form (Toro et al., 1993).

MATERIALS AND METHODS

Coronary smooth muscle membrane vesicles were obtained as described in Toro et al. (1991). Briefly, plasma membrane vesicles from pig coronary smooth muscle were prepared from 20 or 30 arteries. Microsomes were obtained in the presence of proteases inhibitors and were subsequently purified in a sucrose gradient. Membranes obtained from the 20%:25% and 25%:30% (w/w) sucrose interface were used. Lipid bilayers were cast from a phospholipid solution in n-decane containing a 5:2:3 mixture of phosphatidylethanolamine/phosphatidylserine/phosphatidylcholine (25 mg/ml). The voltage control side was the *cis* chamber, and the *trans* chamber was referred to ground. Membrane vesicles were applied on top of the preformed bilayer from the *cis* side. The laterality of channel incorporation was determined by the voltage dependence of channel gating.

The effect of niflumic acid was tested on reconstituted K_{Ca} channels from coronary smooth muscle with low open probability (≤0.25). The experimental solutions were, for the *cis* chamber (mM): 250 KCl, 10 MOPS, 1 HEDTA, 0.67 $CaCl_2$ (pH 7.2, pCa 5.17); for the *trans* chamber (mM): 5 KCl, 245 NaCl, 10 MOPS, 1 HEDTA, 0.67 $CaCl_2$ (pH 7.20, pCa 5.17). Variations to these solutions are indicated in the figure legends. Calcium-activation curves were constructed by perfusing solutions with different [Ca^{2+}]i, calculated according to Fabiato (1988). A rapid perfusion system for a bilayer setup (≈15–30 s) was used to exchange solutions.

Data were acquired on line at 1 ms/point and filtered at 500 Hz using an 8-pole Bessel filter. Analysis was performed using TRANSIT (A. M. J. VanDongen, Duke University, Chapel Hill, NC). Open probability was obtained from the ratio between the open time and the total time. Kinetic analysis was performed in bilayers with a single channel. A critical closed time of 1 s was used to obtain charybdotoxin-induced blocked and unblocked times. Values are means ± SEM. A one-tailed Student's t-test, or ANOVA and multiple comparison Tukey tests were applied; values were considered significantly different at a level $p \leq 0.05$.

Niflumic acid, mefenamic acid, and flufenamic acids were purchased from Sigma Chemical Co. (St. Louis, MO). Concentrated stock solutions (100 mM) of niflumic and flufenamic acids were done in ethanol, whereas mefenamic acid stock solution (100 mM) was prepared in 1:1 ethanol:dimethylsulfoxide (DMSO). Final dilutions had a pH of 7.20 and contained at most 1% ethanol or 0.05% DMSO. These concentrations of solvent did not increase by themselves the channel open probability.

RESULTS AND DISCUSSION

Niflumic acid activates K_{Ca} channels from the external side in a dose-dependent, fast, and reversible manner

The action of niflumic acid on K_{Ca} channels from coronary smooth muscle reconstituted into lipid bilayers was explored.

Received for publication 22 February 1994 and in final form 26 May 1994.

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0006-3495/94/12/2272/08 $2.00

Micromolar concentrations of niflumic acid from the external side caused an increase in channel open probability (P_o) (60 out of 64 studied channels). For example, in 10 experiments with 100 μM niflumic acid channel P_o increased from 0.09 ± 0.01 to 0.35 ± 0.05. When dose-response curves were constructed, the K_d was 261 ± 73 μM (n = 5) and the Hill coefficient approximated 1 (0.99 ± 0.12). Fig. 1 *A* exemplifies a channel with a K_d of 132 μM and a Hill coefficient of 0.6. A Hill coefficient near one indicates that the potentiation of K_{Ca} channels by niflumic acid involves a bimolecular interaction between the drug and its binding site. The stimulatory effect of externally applied niflumic acid was also observed in K_{Ca} channels from other sources like uterine and tracheal smooth muscle, *slo* K_{Ca} channel expressed in oocytes and from skeletal muscle. For example, 100 μM niflumic acid caused an increase in channel P_o from the first three sources from 0.37 to 0.71, from 0.3 to 0.8, and from 0.21 to 0.55, respectively.

Niflumic acid action took place within the time resolution of our perfusion system (15–30 s), and its effect was readily washed out ($P_{o\text{-control}}$ = 0.05 ± 0.01; $P_{o\text{-niflumic acid}}$ = 0.32 ± 0.07; $P_{o\text{-washout}}$ = 0.047 ± 0.01; n = 3) even if a high dose of niflumic acid was used. Fig. 1 *B* shows the time course of two such experiments where 1 mM niflumic acid was perfused to the external side of the channel. The upper graph illustrates the activity of a channel under control conditions ($[Ca^{2+}]_i$ = 7 μM), after diminution of channel activity by lowering $[Ca^{2+}]$i to 3 μM with a calcium chelator (HEDTA), and its fast increase after external perfusion of niflumic acid. The lower panel illustrates in another experiment that niflumic acid-induced increase of K_{Ca} channel activity could be readily reversed after washing out the drug. It seems, therefore, that K_{Ca} channels possess a specific niflumic acid receptor. Furthermore, it is very likely that this receptor is located in the channel protein and not in a closely associated molecule because we have observed that external niflumic is also able to stimulate a cloned K_{Ca} channel reconstituted in lipid bilayers (Pérez et al., 1994; our unpublished observations).

Mechanism of niflumic acid activation of K_{Ca} channels

Because K_{Ca} channels are voltage- and calcium-sensitive, we decided to study whether niflumic acid affected these channel properties. We found that niflumic acid from the external side exerted its effect on K_{Ca} channels by left-shifting both their voltage- and calcium-activation curves without major changes in the slopes of the curves. Fig. 2 *A* shows that perfusion of 100 μM niflumic acid to the external side in-

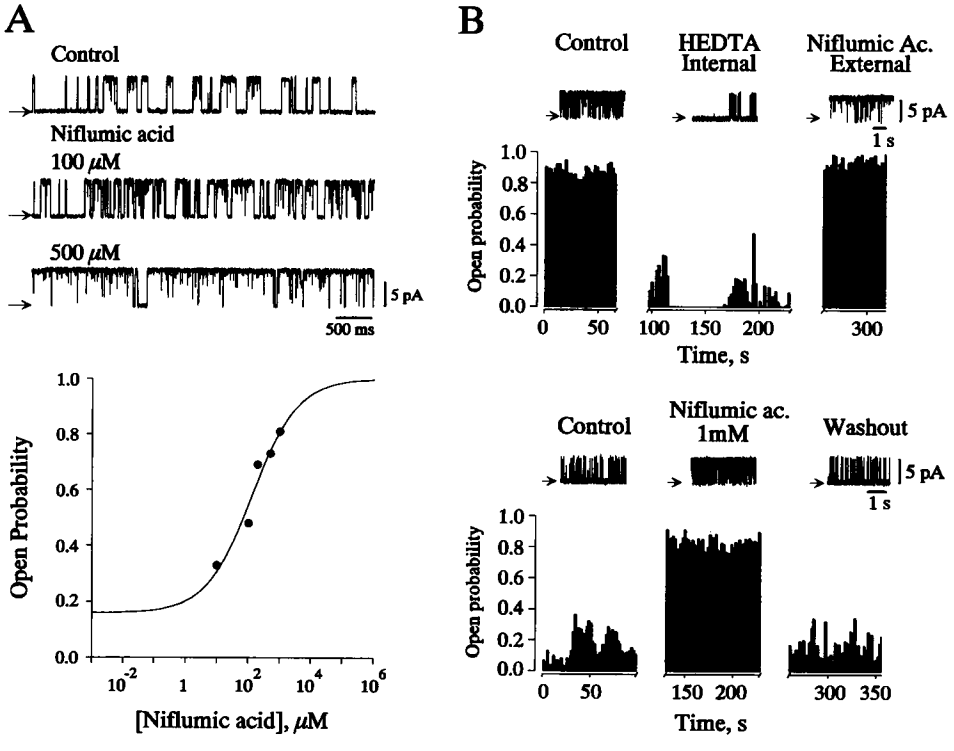

FIGURE 1 Niflumic acid activates K_{Ca} channels. The action of external niflumic acid on Ca^{2+}-activated K^+ (K_{Ca}) channels was assessed using pig coronary smooth muscle plasma membranes incorporated into lipid bilayers. (*A*) Records illustrating K_{Ca} channel activation by niflumic acid at different [niflumic acid], and corresponding dose-response curve. Experimental data were fitted to: Normalized $P_o = (1 - A)/(1 + (K_{1/2}/[\text{niflumic acid}])^N) + A$, where $K_{1/2}{}^N = K_d$ and A is the channel P_o (0.16) before addition of niflumic acid. Fitted values were K_d = 132 μM and N = 0.6. (*B*) Fast activation of a K_{Ca} channel by niflumic acid (1 mM) (upper panel). $P_{o\text{-control}}$ = 0.87 ($[Ca^{2+}]$i = 7 μM); $P_{o\text{-HEDTA}}$ = 0.07 ($[Ca^{2+}]$i = 3 μM), and after external perfusion of 1 mM niflumic acid $P_{o\text{-niflumic acid}}$ = 0.93. The lower panel shows in another experiment the reversibility of niflumic acid effect. $P_{o\text{-control}}$ = 0.15, P_o after 1 mM niflumic acid was 0.82, and P_o after washout was 0.15. V_H = 0 mV. Brakes in the time axes correspond to the perfusion time. Arrows indicate the closed state of the channel.

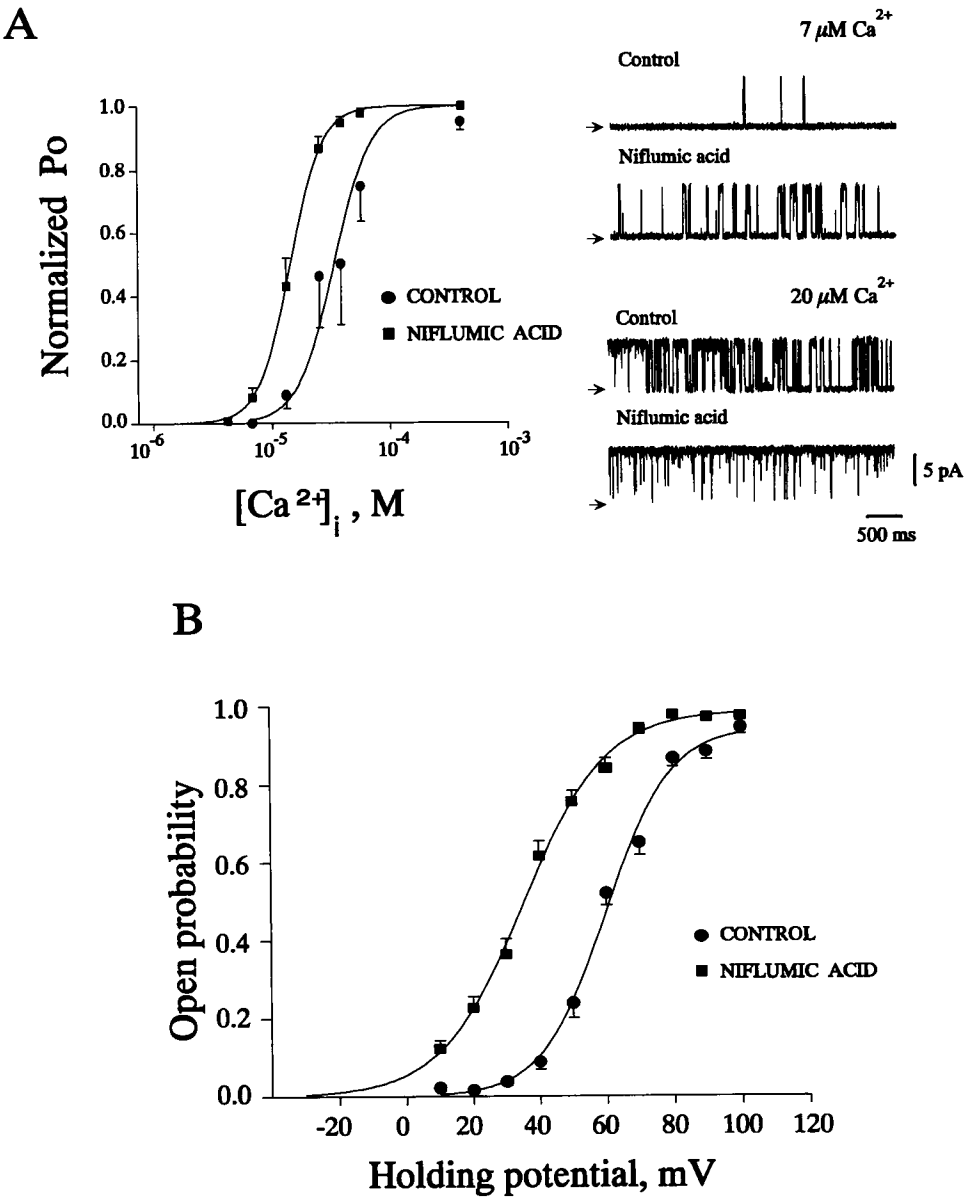

FIGURE 2 Niflumic acid causes a negative shift in both calcium and voltage activation curves. (*A*) (*left*) Calcium-activation curves before (●) and after niflumic acid (■). Channel activity was recorded for 2–5 min at each calcium concentration. Experimental values were normalized with respect to the maximum P_o attained in each experiment (ranged from 0.96 to 0.98) in the presence of 100 μM niflumic acid and 400 μM Ca^{2+}. Each point is the mean of four experiments (bars represent SEM). Data were fitted using: Normalized $P_o = 1/(1 + (K_{1/2}/[Ca^{2+}]_i)^N)$. $K_{1/2}$ in the control was 32 ± 2.6 μM, and after niflumic acid $K_{1/2}$ diminished to 14 ± 0.1 μM. The Hill coefficient did not change significantly after niflumic acid (2.1 ± 0.4 vs. 3.4 ± 0.08, $p = 0.05$). (*right*) Examples of channel activity before and after 100 μM niflumic acid at two $[Ca^{2+}]i$, 7 and 20 μM. P_o values were 0.002 and 0.72 in control and 0.17 and 0.94 after niflumic acid, respectively. $V_H = 0$ mV. (*B*) Voltage activation curves of the same channel before (●) and after (■) niflumic acid. Points are the mean values for 6 and 9 ramps, respectively. Voltage ramps from 0 to 100 mV of 2 s duration were used. To take into account any possible spontaneous variation in open probability during time, we obtained sets (3 ramps each) of ramps with 3 min intervals. P_o values were calculated after leakage correction and from idealized records setting a threshold to the half amplitude as a function of the potential. P_o was calculated as the open time divided by the total time every 10 mV. The voltage values in the graph correspond to the middle voltage in every 10 mV segment. Data were fitted to a Boltzmann distribution (——): $P_o = 1/(1 + \exp((V_{1/2} - V)/k))$, where $V_{1/2}$ is the half-activation potential, k (slope factor) = RT/zF, z is the effective valence, and R, T, and F have their usual thermodynamic meanings. Control $V_{1/2}$ was 62 ± 0.9 mV, and after external niflumic ac. was 36 ± 0.5 mV. The slope factor was practically the same in both conditions (11 ± 0.8 vs. 13 ± 0.5 mV). Symmetrical 250 mM KCl. In *A* and *B*, [Niflumic acid] = 100 μM.

creased channel affinity for Ca^{2+} from a $K_{1/2}$ of 32 ± 2.6 μM to a $K_{1/2}$ of 14 ± 0.1 μM ($n = 4$). In another two channels, the mean $K_{1/2}$ for Ca^{2+} was decreased from 11 to 7.3 μM. In all experiments, the Hill coefficient did not vary significantly (3.1 ± 0.5 vs. 3.8 ± 0.4, $n = 6$). On the other hand, niflumic acid shifted the half-activation potential ($V_{1/2}$) to more negative values by 30 ± 3 mV, whereas the slope factor (k) remained unchanged (12 ± 0.5 mV) ($n = 3$). This is exemplified in Fig. 2 *B* where niflumic acid diminished $V_{1/2}$ from 62 to 36 mV. These experiments are consistent with the idea that the calcium site of K_{Ca} channels senses the voltage and demonstrates that the opening of K_{Ca} channels by ni-

flumic acid is caused by an increase in the sensitivity of channel gating to calcium.

One explanation to the shift in voltage caused by niflumic acid is that the anionic form of niflumic acid exerts a general nonspecific surface charge effect on the external side of the membrane that results in a local depolarization and a concomitant increase in channel activity (Hille, 1993). A similar change in local potential can be achieved by a *specific* binding of niflumic acid to its receptor increasing the local negative charge density near the channel voltage sensor, causing a shift in its voltage dependence. A comparable mechanism has been proposed for phosphorylation of a delayed rectifier K^+ channel (Perozo and Bezanilla, 1990). We favor the notion of a specific binding of niflumic acid to its receptor because, as shown later, the analog mefenamic acid, which is also negatively charged, is less effective than niflumic acid. Furthermore, a nonselective local depolarization of the lipid bilayer cannot explain the lack of a stimulatory effect of 1 mM niflumic acid on cardiac Ca^{2+} channels and *Shaker* K^+ channels expressed in oocytes (our unpublished observations), nor its inhibitory effect on Ca^{2+}-dependent Cl^- channels (White and Aylwin, 1990).

Kinetic analysis showed that niflumic acid-induced activation of K_{Ca} channels causes a marked decrease in the channel mean closed time (τ_c), whereas the mean open time (τ_o) slightly increased or was practically unchanged. For example, a large dose of niflumic acid (1 mM) only increased τ_o from 26 ± 5 to 57 ± 2 ms, whereas τ_c was dramatically reduced from 289 ± 68 ms to 14 ± 1 ms (n = 4). Fig. 3 *A* illustrates the distribution of open and closed dwell-times at two niflumic acid concentrations (100 μM and 1 mM), Fig. 3 *B* are the time constant vs. [niflumic acid] plots, and Fig. 3 *C* compares the relative contribution of each closed time constant for the same experiment. Open times could be fitted to a single exponential function, whereas the closed times were multiexponential. Note that in this channel the mean open time was essentially unaffected by niflumic acid; in contrast, closed times, τ_{c2} and τ_{c3}, were largely diminished by the drug. The longest closed time, τ_{c3}, not only diminished in magnitude, but this type of event almost vanished after 1 mM niflumic acid (values are in the legend). Thus, niflumic acid opens maxi K_{Ca} channels mainly by decreasing the time the channels stay in the longer closed states.

The external receptor of niflumic acid is not within the conduction pathway of K_{Ca} channels

Tetraethylammonium (TEA) and charybdotoxin (CTX) are known to interact with the external vestibule of K channels occluding K flux through the pore (Yellen, 1984; Miller

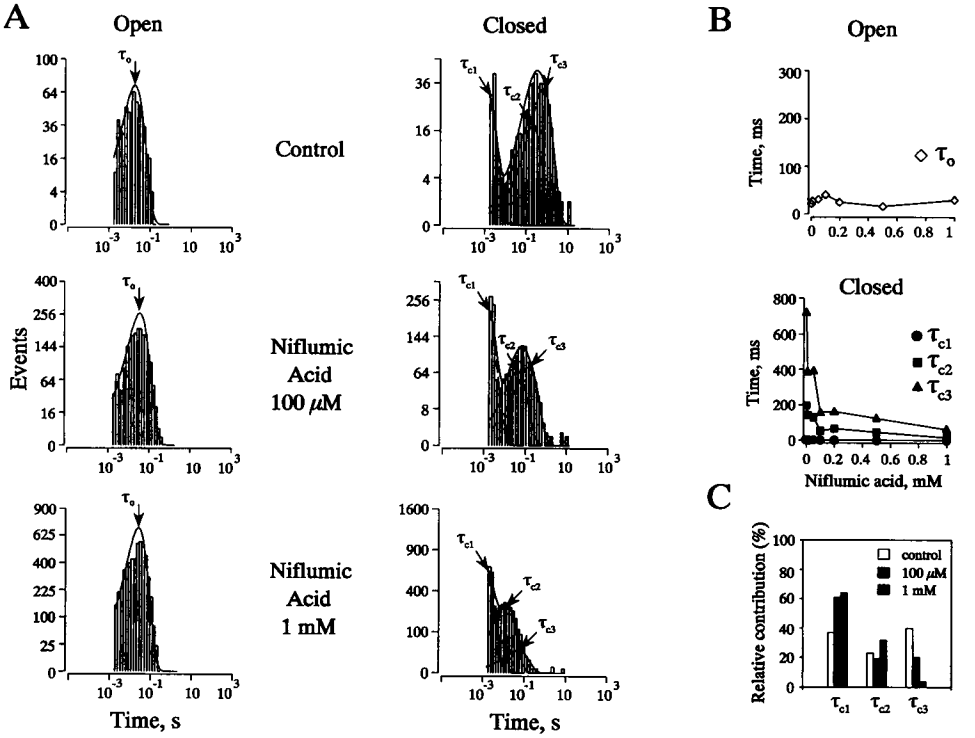

FIGURE 3 Kinetics of niflumic acid-induced activation of maxi K_{Ca} channels. (*A*) Dwell-time histograms of open (τ_o) and closed times (τ_c) before (control) and after addition of 100 μM and 1 mM niflumic acid. Values were for τ_o: 22 ms (control, # events = 538), 41 ms (100 μM, # events = 2041), and 30 ms (1 mM, # events = 5338). Closed times were for τ_{c1}: 1.4 ms (control), 1.5 ms (100 μM), and 1.9 ms (1 mM); for τ_{c2}: 197 ms (control), 52 ms (100 μM) and 16 ms (1 mM); for τ_{c3}: 724 ms (control), 166 (100 μM) and 69 (1 mM). The value of τ_{c1} is underestimated because acquisition was made at 1 ms/point. % values are the percentage of the total # of events that contributed to that particular kinetic component. Events larger than ≈2 s duration were left out of the fit because they were too few to be considered. Histograms were logarithmically binned, and the peaks correspond to the time constants. Continuous lines are the sum of the individual components (----) of the probability density function fitted after correcting for death time. (*B*) Open and closed times as a function of niflumic acid concentration. (*C*) Relative contribution of each closed time constant before (control), and after 100 μM and 1 mM niflumic acid. *A*, *B*, and *C* correspond to the same channel.

et al., 1985; MacKinnon and Miller, 1988). In particular, K_{Ca} channel proteins have been shown to possess a specific TEA receptor in their outer mouth (Latorre et al., 1989; Adelman et al., 1992), whereas studies in native, purified, and cloned K_{Ca} channels indicate that the receptor for CTX is localized in the extracellular domain of the channel protein (Miller, 1988; Butler et al., 1993; García-Calvo et al., 1994; Stampe et al., 1994). To investigate whether niflumic acid acts in or nearby the external side of the conduction pathway, we explored whether the binding with its receptor modifies K flux or TEA and CTX blockade.

The fast channel blockade induced by externally applied TEA (reduction in channel amplitude) was not affected by previous or subsequent addition of niflumic acid. In experiments where niflumic acid was added before TEA, application of TEA produced the expected reduction in channel amplitude and caused a slight decrease in channel open probability ($n = 4$). For example, in two experiments 160 μM TEA diminished channel P_o about 20%. This decrease in P_o is most probably caused by the inherent P_o reduction observed in coronary smooth muscle K_{Ca} channels with TEA alone (Toro et al., 1991) (Fig. 4 *A*, *middle* panel), and not to a TEA competition with or destabilization of niflumic acid binding to its receptor. Accordingly, Fig. 4 *A* illustrates that the blockade of a K_{Ca} channel by externally applied TEA (160 μM) did not prevent its potentiation by niflumic acid (10 μM). Similar results were obtained in another four experiments using 150–200 μM TEA and stimulating channel activity with 25 to 100 μM niflumic acid. These results strongly suggest that TEA and niflumic acid do not interact with the same site.

Consistent with the above conclusion, CTX, which is known to compete with TEA (Miller, 1988), did not compete with niflumic acid. If niflumic acid were to compete with CTX for the same site the k_{on} of CTX should decrease in the presence of niflumic acid. The experiment in Fig. 4 *B* shows that this is not the case and supports the idea that niflumic acid binding site is not the same as the one for CTX. Note, however, that as demonstrated by Anderson et al. (1988) for Ca^{2+}, CTX binds faster to the open than to the closed channel; thus, an increase in the burst P_o by 300 μM external niflumic acid (burst Po increased from 0.86 to 0.94) was followed by a concomitant increase in the k_{on} of CTX (k_{on} increased from 6×10^6 s^{-1} M^{-1} in the absence of niflumic acid to 9×10^6 s^{-1} M^{-1} after niflumic acid). Accordingly, in another experiment, when the burst Po was 0.2 the k_{on} for

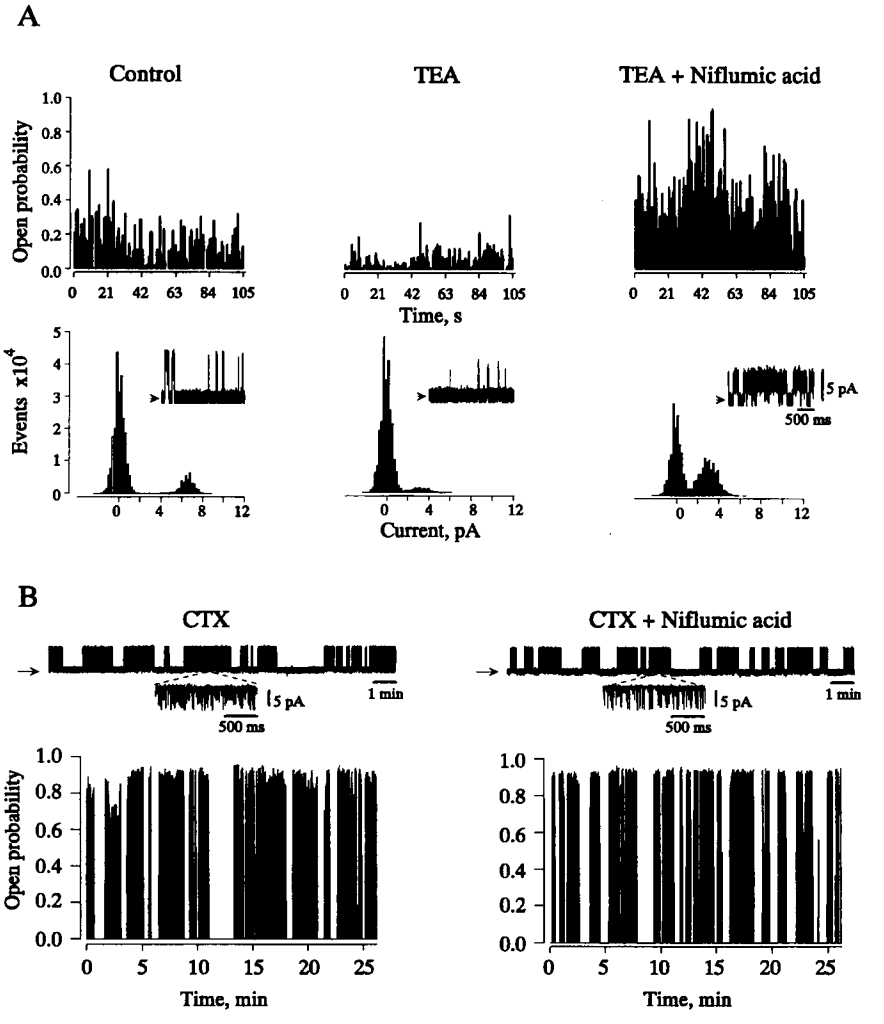

FIGURE 4 Niflumic acid action does not interfere with external TEA or CTX channel blockade. (*A*) P_o vs. time graphs and total point histograms of an experiment where TEA blockade was followed by niflumic acid-induced potentiation of channel activity. Insets are examples of channel records under each experimental condition. Mean P_o values were: control, 0.14; after 160 μM external TEA, 0.05, and after 10 μM external niflumic acid, 0.43. Note that external TEA reduced channel amplitude by about half its initial value as expected for a fast blockade. Channel amplitude remained the same after niflumic acid activation, as shown in the total point histograms. Histograms were fitted to a double Gaussian function. Fitted channel amplitude values were: Control 6.5 ± 0.12 pA, after TEA 3.1 ± 1.6 pA, and after niflumic acid 3 ± 0.12 pA. $V_H = 0$ mV. (*B*) Channel traces and P_o vs. time plots of the same channel in the presence of 5 nM CTX and after addition of 300 μM niflumic acid to the external side. Niflumic acid did not increase the unblocked times ($\tau_{u\text{-}CTX} = 33 \pm 7$ s vs. $\tau_{u\text{-}CTX+Niflumic} = 22 \pm 2$ s) $\tau_u = \{k_{ON}*[CTX]\}^{-1}$ and the CTX-induced blocked times did not change significantly after niflumic acid addition ($\tau_{b\text{-}CTX} = 20 \pm 5$ s vs. $\tau_{b\text{-}CTX+Niflumic} = 19 \pm 4$ s), discarding a competitive interaction. Note that the unblocked times slightly decreased after addition of niflumic acid as expected for the preference of CTX to bind to the open channel (see text). Burst P_o increased from 0.86 (*left*) to 0.94 after niflumic acid (*right*). Mean channel P_o (averaged over the whole trace) was 0.53 with CTX and 0.49 with CTX + niflumic acid.

CTX was 7.7×10^5 s^{-1} M^{-1} and changed to 14.5×10^5 s^{-1} M^{-1} when the burst P_o was increased to 0.68 with niflumic acid. On the other hand, a channel first activated with 200 μM niflumic acid (average $P_{o\text{-control}} = 0.02$; average $P_{o\text{-niflumic acid}} = 0.76$) was readily blocked by subsequent addition of 4 nM CTX (P_o averaged over the whole record diminished to 0.04). Qualitatively similar results were obtained in another 4 experiments. Furthermore, elevating internal [K^+] to favor K outflux did not destabilize the interaction of niflumic acid with its external side, because channels remained activated ($n = 3$).

Taken together, these results strongly suggest that the receptor for niflumic acid is not located at or near the pore of the maxi K_{Ca} channel and that niflumic acid association to its receptor does not alter the functional properties of TEA and CTX receptors located in the external vestibule of the channel pore.

Analogs of niflumic acid also stimulate K_{Ca} channel activity from the external side

Fig. 5 shows that compounds with similar chemical structure (panel A) than niflumic acid can also increase K_{Ca} channel activity, namely, flufenamic and mefenamic acids. Similarly to niflumic acid, the stimulatory action of these analogs was reversible. Fig. 5 *B* are examples of different channels activated by 100 μM of each of the three analogs. As quantified in Fig. 5 *B*, flufenamic and niflumic acids had similar potencies, whereas mefenamic acid was the less effective activator of maxi K_{Ca} channels. Mean values for the normalized fraction of increase were: for mefenamic acid, 0.09 ± 0.02, $n = 12$; for niflumic acid, 0.28 ± 0.05, $n = 12$, and for flufenamic acid, 0.37 ± 0.08, $n = 6$. Consistent with this affinity sequence (flufenamic ac. ≈ niflumic ac. ≫ mefenamic ac.), large concentrations of mefenamic acid (1 mM, $n = 3$) had to be used to obtain a similar activation as the one observed with 100 μM niflumic acid. Furthermore, when all three compounds were tested on the same channel, similar results were obtained ($n = 2$).

It is interesting to note that niflumic and flufenamic acids, effective K_{Ca} channel activators, have in common a —CF_3 group that is absent in mefenamic acid, a drug with limited stimulatory properties (Fig. 5 *A*). This polar group may be an important structural factor in the action of these N-arylated derivatives leading to an increase in channel activity. In addition, the fact that mefenamic acid a compound with higher hydrophobicity (Dhanaraj and Vijayan, 1988) (two methyl groups instead of a polar —CF_3, see Fig. 5 *A*) was less potent in increasing K_{Ca} channel activity supports the idea that activation of K_{Ca} channels by niflumic acid involves a specific interaction with its receptor and is not caused by a nonspecific effect in the lipid bilayer.

K_{Ca} channels are activated by internal fenamates less effectively

To determine whether K_{Ca} channels can be activated by internally applied niflumic acid, we performed a series of experiments where we recorded channel activity immediately after internal perfusion of the drug and tested the reversibility of the effect. As illustrated in Fig. 6 *A*, internal perfusion of niflumic acid (*left* traces) also promoted channel activation of K_{Ca} channels but, in general, a higher dose of the drug was necessary to reach a similar activity when compared with external perfusion (*right* traces). This effect of internal niflumic acid was quickly established and was also washed out

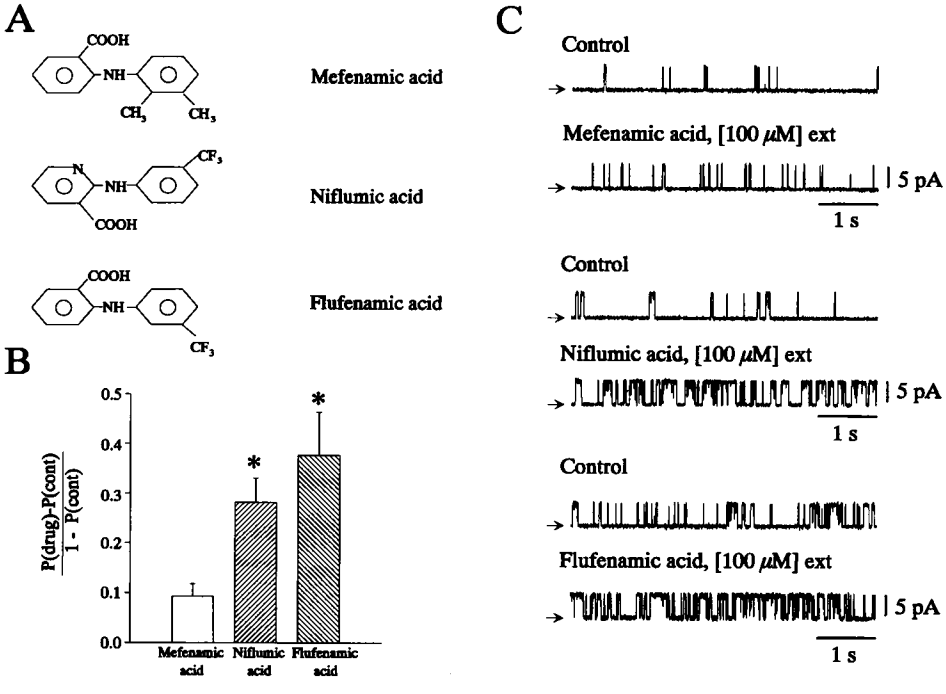

FIGURE 5 Analogs of niflumic acid can increase channel activity. (*A*) Chemical structures of mefenamic, flufenamic and niflumic acids. (*B*) The bar plot shows the mean of the normalized fraction of increase obtained with 100 μM of each compound. Mean values were: for mefenamic acid, 0.09 ± 0.02, $n = 12$; for niflumic acid, 0.28 ± 0.05, $n = 12$, and for flufenamic acid, 0.37 ± 0.08, $n = 6$. Bars are SEM. ANOVA at $p = 0.05$ followed by multiple comparison Tukey test ($p = 0.05$) determined that niflumic and flufenamic acids (*) were significantly more potent activators of maxi K_{Ca} channels than mefenamic acid. Niflumic and flufenamic acids potencies were not significantly different ($p = 0.05$). (*C*) Effect of mefenamic, niflumic, and flufenamic acids on different channels (100 μM). Mean P_o values in 2 min recordings at 0 mV were: 0.01 control and 0.08 after mefenamic ac.; 0.05 control and 0.36 after niflumic ac., and 0.15 control and 0.56 after flufenamic ac. $V_H = 0$ mV.

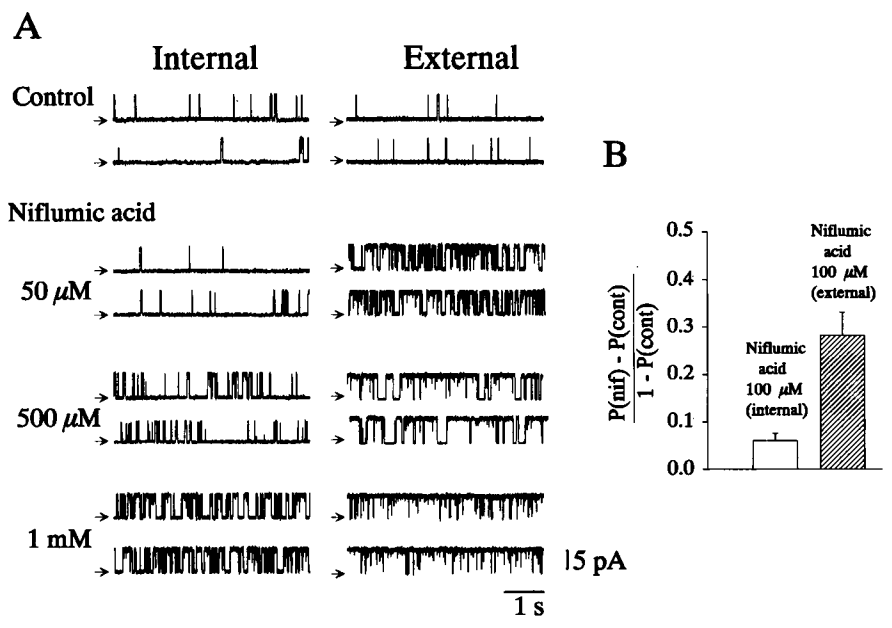

FIGURE 6 Niflumic acid increases K_{Ca} channel activity preferentially from the external side. (*A*) Records show channel activity in presence of internal (*left* traces) or external (*right* traces) niflumic acid at different concentrations. Notice that at the same concentration of niflumic acid the increase in the channel activity was less when the drug was perfused in the internal side. V_H = 0 mV. (*B*) Normalized fraction of increase by 100 μM niflumic acid from the internal (*left* bar) or external (*right* bar) side of the channel. Normalized fraction of increase was significantly different, 0.06 ± 0.01 (n = 14) vs. 0.28 ± 0.05 (n = 12) with internal and external niflumic acid, respectively.

(n = 6) within the time-resolution of our perfusion system (15–30 s). As quantified in Fig. 6 *B*, 100 μM niflumic acid was much more effective when applied from the external side of the channel than from the internal side. Normalized fraction of increase was 0.06 ± 0.01 (n = 14) vs. 0.28 ± 0.05 (n = 12) with internal and external niflumic acid, respectively. This differential action was further established when we tested niflumic acid from both sides in the same channel. In two successful experiments, *external* perfusion of the drug was followed by washout and recovery, before addition of *internal* niflumic acid or vice versa. The normalized fraction of increase by 100 μM niflumic acid was 0.08 ± 0.04 (internal) and 0.36 ± 0.15 (external).

The differential action of niflumic acid from the external versus the internal side was also observed when flufenamic and mefenamic acids were tested. The potency of 100 μM of these derivatives from the internal side were for flufenamic 0.11 ± 0.04 (normalized fraction of increase, n = 5) and for mefenamic acids 0.03 ± 0.008 (n = 8) vs. 0.37 ± 0.08 and 0.09 ± 0.02 for external flufenamic and mefenamic acids, respectively (see Fig. 5 for comparison). In a small population of channels (3 out of 23), internal application of fenamates produced a larger degree of activation. The normalized fraction of increase was 0.45 ± 0.04 for niflumic acid, 0.44 ± 0.07 for flufenamic acid, and 0.11 ± 0.02 for mefenamic acid. This higher sensitivity of the internal side could be explained by the presence of a relatively small population of another K_{Ca} channel isoform with an increased affinity for internal and external niflumic acid in our preparation, or else caused by a modification of the channel properties during isolation.

We have shown that niflumic acid applied from the internal side of K_{Ca} channels was less potent than when applied from the external side. In both cases, their stimulatory action was dose-dependent, rapid, and reversible. At least two explanations are possible: 1) that internal niflumic acid rapidly diffuses through the membrane and that its stimulatory effect from the internal side is caused by the occupancy of an external receptor, and 2) that K_{Ca} channels possess both external and internal receptors with different affinities for niflumic acid. Because niflumic acid is an amphipathic molecule (Dhanaraj and Vijayan, 1988), it is possible that its receptor is conformed by an hydrophobic "pocket" facing the aqueous phase. Structure-function experiments should answer these questions.

In summary, niflumic acid activates maxi K_{Ca} channels from both their external and internal sides, being more efficacious from their external side. Our results support the idea that K_{Ca} channels possess at least one fenamate receptor and indicate that this receptor is not located at or in the vicinity of TEA and CTX receptors of the maxi K_{Ca} channels. Finally, niflumic acid may be a prototype of K_{Ca} channel agonists with potential therapeutic implications in smooth muscle, where these channels play an important physiological role, and may help to map functional properties of maxi K_{Ca} channels.

We would like to thank Dr. E. Stefani for helpful discussion, Dr. R. Olcese for initial experiments, Dr. A. M. J. VanDongen for TRANSIT, and Mr. Garland Cantrell for the bilayer amplifier.
This work was supported by grant HL47382 (L. Toro).

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